

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

Kreisler 1098-KGB

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

09/914658

INTERNATIONAL APPLICATION NO.

PCT/EP00/01903

INTERNATIONAL FILING DATE

3 March 2000 (03.03.00)

PRIORITY DATE CLAIMED

6 March 1999 (06.03.99)

TITLE OF INVENTION

STABLE RECOMBINANT INFLUENZA VIRUSES FREE OF HELPER VIRUSES

APPLICANT(S) FOR DO/EO/US

GERD HOBOM, RAMON FLICK, ANETTE MENKE and MAYSA AZZEH

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ Certificate of Mailing by Express Mail
20. ☒ Other items or information:

-Statement Regarding Sequence Listing
-Disk containing Sequence Listing in Word Format
-Express Mail Certificate
-Return Receipt Postcard

U.S. APPLICATION NO. 09/914658	INTERNATIONAL APPLICATION NO. PCT/EP00/01903	ATTORNEY'S DOCKET NUMBER Kreisler 1098-KGB
---------------------------------------	--------------------------------------------------------	------------------------------------------------------

21. The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

- ☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **\$1,000.00**
- ☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **\$860.00**
- ☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **\$710.00**
- ☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **\$690.00**
- ☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **\$100.00**

ENTER APPROPRIATE BASIC FEE AMOUNT =Surcharge of **\$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total claims	30 - 20 =	10	x \$18.00
Independent claims	1 - 3 =	0	x \$80.00

Multiple Dependent Claims (check if applicable). ☐**TOTAL OF ABOVE CALCULATIONS =**Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) (check if applicable). ☐**SUBTOTAL =**Processing fee of **\$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).**TOTAL NATIONAL FEE =**Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable). ☐**TOTAL FEES ENCLOSED =**

Amount to be:	\$
refunded	
charged	\$

- ☐ A check in the amount of _____ to cover the above fees is enclosed.
- ☒ Please charge my Deposit Account No. **14-1263** in the amount of **\$1,040.00** to cover the above fees.
A duplicate copy of this sheet is enclosed.
- ☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **14-1263**. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Norris McLaughlin & Marcus
220 East 42nd Street
30th Floor
New York, New York 10017

SIGNATURE

Kurt G. Briscoe

NAME

33,141

REGISTRATION NUMBER

8-31-01

DATE

Kreiser 1098-KGB

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

09/914658

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371INTERNATIONAL APPLICATION NO.
PCT/EP00/01903INTERNATIONAL FILING DATE
3 March 2000 (03.03.00)PRIORITY DATE CLAIMED
6 March 1999 (06.03.99)

TITLE OF INVENTION

STABLE RECOMBINANT INFLUENZA VIRUSES FREE OF HELPER VIRUSES

APPLICANT(S) FOR DO/EO/US

GERD HOBOM, RAMON FLICK, ANETTE MENKE and MAYSA AZZEH

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ Certificate of Mailing by Express Mail
20. ☒ Other items or information:

-Statement Regarding Sequence Listing
-Disk containing Sequence Listing in Word Format
-Express Mail Certificate
-Return Receipt Postcard

009/91465821502
518 Rec'd PCT/PTO 31 AUG 2001

Express Mail Label No. EL 867734761 US
Date Mailed: August 31, 2001

Kreisler 1098-KGB
012142us/JH/ml

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS : GERD HOBOM, RAMON FLICK, ANETTE MENKE and MAYSA
AZZEH
SERIAL NO. : TO BE ASSIGNED (International Application No.: PCT/EP00/01903)
FILED : HEREWITH
FOR : STABLE RECOMBINANT INFLUENZA VIRUSES FREE OF
HELPER VIRUSES
ART UNIT : TO BE ASSIGNED
EXAMINER : TO BE ASSIGNED

August 31, 2001

Hon. Commissioner of Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

SIR:

Prior to examination, please amend the above-identified application as follows:

IN THE SPECIFICATION:

Insert the following new paragraph as the first paragraph of the application: -- This application
is a 371 of PCT/EP00/01903 filed on March 3, 2000. --

IN THE CLAIMS:

Please amend claims 3, 5, 10-23 and 25-30 as follows:

3. The recombinant influenza virus according to claim 1, wherein one or more of the regular viral RNA segments, differing from said at least one ambisense RNA segment, comprises a vRNA encoding a foreign gene, preferably one or more of the regular viral RNA segments has (have) been exchanged for a vRNA encoding a foreign gene.

5. The recombinant influenza virus according to claim 1, in which the terminal viral RNA sequences of one or more of the regular segments and/or of the at least one ambisense RNA segment, which are active as the promoter signal, have been modified by nucleotide substitutions in up to five positions, resulting in improved transcription rates of both the vRNA promoter as well as the cRNA promoter as present in the complementary sequence.

10. The recombinant influenza virus of claim 6, wherein the 5' terminal nucleotide sequence comprises the modifications U3A and A8U resulting in a 5'-terminal sequence of 5'-AGAAGAAUCAAGG.

11. The recombinant influenza virus according to claim 1, which is a recombinant influenza A virus.

12. The recombinant influenza virus according to claim 1, in which the foreign gene(s) in ambisense covalent junction with viral gene(s) code for proteins and/or glycoproteins which are secreted from cells infected with the recombinant virus.

13. The recombinant virus according to claim 1, in which the foreign gene(s) in ambisense covalent junction with viral gene(s) code for proteins or artificial polypeptides designed to support an efficient presentation of inherent epitopes at the surface of infected cells, for stimulation of a B cell and/or T cell response.
14. A method for the production of recombinant influenza viruses as defined in claim 1 comprising
- (a) RNA polymerase I synthesis of recombinant vRNAs *in vivo*, in ambisense design,
 - (b) followed by infection with an influenza carrier strain constructed to include flanking ribozyme target sequences in at least one of its viral RNA segments which is (are) to be replaced by the ambisense segments of step (a), and
 - (c) thereafter selective vRNA inactivation through ribozyme cleavage.
15. A pharmaceutical composition comprising a recombinant influenza virus according to claim 1.
16. Method of using a medicament comprising a recombinant influenza virus according to claim 1 for vaccination purposes.
17. The method according to claim 16, wherein the medicament
- (a) is suitable against influenza and/or against other infections;

(b) is present in form of inactivated preparations; and/or

(c) is present in form of live recombinant viruses.

18. Method of using an agent comprising a recombinant influenza virus according to claim 1 for somatic gene therapy.

19. Method of using an agent comprising a recombinant influenza virus according to claim 1 for transfer and expression of foreign genes into cells infected by such viruses.

20. Method of using an agent comprising a recombinant influenza virus according to claim 1 for transfer and expression of RNA molecules into cells infected by such viruses.

21. The method of claim 20, wherein the RNA molecules to be expressed are antisense sequences or double-strand sequences relative to the target cell cellular mRNA molecules, and/or the agent is suitable for sequence-specific gene silencing, preferably by antisense RNA or RNA interference mechanisms.

22. The method according to claim 18, wherein the agent is applicable in *ex vivo* and *in vivo* application schemes.

23. A method for the production of proteins or glycoproteins which comprises utilizing a

recombinant influenza virus according to claim 1 as expression vector.

25. A method for preventing and/or treating influenza which comprises administering an effective amount of a recombinant influenza virus according to claim 1 to the mammal to be treated.
26. A method for somatic gene therapy, which method comprises subjecting the organism to be treated with a recombinant influenza virus according to claim 1.
27. A method for transfer and expression of foreign genes into cells, and for transfer and expression of RNA molecules into cells, which method comprises infecting the cells with a recombinant influenza virus according to claim 1.
28. Method of using an agent comprising a recombinant influenza virus according to claim 1 for autologous immunotherapy.
29. A method for an immunotherapy which comprises *ex vivo* infection of immune cells with a recombinant influenza virus according to claim 1, and introduction of the transduced cells into the patient.
30. A method for the induction of antibodies which comprises utilizing a recombinant

GERD HOBOM ET AL
INTERNATIONAL APPL. NO.: PCT/EP00/01903

influenza virus according to claim 1 as an immunogen.

REMARKS

Applicants intend the examination to begin based on the amended claims filed May 21, 2001.

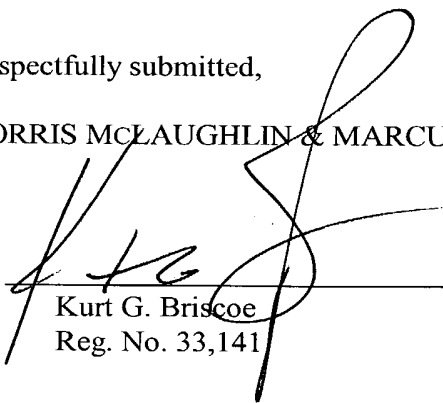
The amendments above remove multiple dependencies to reduce the filing cost, and convert the "use" claims to the conventional "Method of use" claims.

Early and favorable action is earnestly solicited.

Respectfully submitted,

NORRIS McLAUGHLIN & MARCUS, P.A.

By


Kurt G. Briscoe
Reg. No. 33,141

KGB:ja
220 East 42nd Street
30th Floor
New York, New York 10017
(212) 808-0700

MARK-UP SHOWING THE CHANGES MADE IN THE PREVIOUS CLAIM TO YIELD THE CLAIM AS AMENDED ABOVE

3. The recombinant influenza virus according to [claims 1 and 2] **claim 1**, wherein one or more of the regular viral RNA segments, differing from said at least one ambisense RNA segment, comprises a vRNA encoding a foreign gene, preferably one or more of the regular viral RNA segments has (have) been exchanged for a vRNA encoding a foreign gene.
5. The recombinant influenza virus according to [claims 1 to 4] **claim 1**, in which the terminal viral RNA sequences of one or more of the regular segments and/or of the at least one ambisense RNA segment, which are active as the promoter signal, have been modified by nucleotide substitutions in up to five positions, resulting in improved transcription rates of both the vRNA promoter as well as the cRNA promoter as present in the complementary sequence.
10. The recombinant influenza virus of [claims 6 to 9] **claim 6**, wherein the 5' terminal nucleotide sequence comprises the modifications U3A and A8U resulting in a 5'-terminal sequence of 5'-AGAAGAAUCAAGG.
11. The recombinant influenza virus according to [claims 1 to 10] **claim 1**, which is a recombinant influenza A virus.
12. The recombinant influenza virus according to [claims 1 to 11] **claim 1**, in which the

foreign gene(s) in ambisense covalent junction with viral gene(s) code for proteins and/or glycoproteins which are secreted from cells infected with the recombinant virus.

13. The recombinant virus according to [claims 1 to 11] **claim 1**, in which the foreign gene(s) in ambisense covalent junction with viral gene(s) code for proteins or artificial polypeptides designed to support an efficient presentation of inherent epitopes at the surface of infected cells, for stimulation of a B cell and/or T cell response.

14. A method for the production of recombinant influenza viruses as defined in [claims 1 to 13] **claim 1** comprising

- (a) RNA polymerase I synthesis of recombinant vRNAs *in vivo*, in ambisense design,
- (b) followed by infection with an influenza carrier strain constructed to include flanking ribozyme target sequences in at least one of its viral RNA segments which is (are) to be replaced by the ambisense segments of step (a), and
- (c) thereafter selective vRNA inactivation through ribozyme cleavage.

15. A pharmaceutical composition comprising a recombinant influenza virus according to [claims 1 to 13] **claim 1**.

16. [Use of] **Method of using a medicament comprising** a recombinant influenza virus according to [claims 1 to 13 for preparing a medicament] for vaccination purposes.

17. The [use] **method** according to claim 16, wherein the medicament
- (a) is suitable against influenza and/or against other infections;
 - (b) is present in form of inactivated preparations; and/or
 - (c) is present in form of live recombinant viruses.
18. [Use of] **Method of using an agent comprising** a recombinant influenza virus according to [claims 1 to 13 for preparing agents] **claim 1** for somatic gene therapy.
19. [Use of] **Method of using an agent comprising** a recombinant influenza virus according to [claims 1 to 13 for preparing agents,] **claim 1** for transfer and expression of foreign genes into cells infected by such viruses.
20. [Use of] **Method of using an agent comprising** a recombinant influenza virus according to [claims 1 to 13 for preparing agents] **claim 1** for transfer and expression of RNA molecules into cells infected by such viruses.
21. The [use] **method** of claim 20, wherein the RNA molecules to be expressed are antisense sequences or double-strand sequences relative to the target cell cellular mRNA molecules, and/or the agent is suitable for sequence-specific gene silencing, preferably by antisense RNA or RNA interference mechanisms.

22. The [use] **method** according to [claims 18 to 21] **claim 18**, wherein the agent[s are] **is** applicable in *ex vivo* and *in vivo* application schemes.
23. A method for the production of proteins or glycoproteins which comprises utilizing a recombinant influenza virus according to [claims 1 to 13] **claim 1** as expression vector.
25. A method for preventing and/or treating influenza which comprises administering an effective amount of a recombinant influenza virus according to [claims 1 to 13] **claim 1** to the mammal to be treated.
26. A method for somatic gene therapy, which method comprises subjecting the organism to be treated with a recombinant influenza virus according to [claims 1 to 13] **claim 1**.
27. A method for transfer and expression of foreign genes into cells, and for transfer and expression of RNA molecules into cells, which method comprises infecting the cells with a recombinant influenza virus according to [claims 1 to 13] **claim 1**.
28. [Use of] **Method of using an agent comprising** a recombinant influenza virus according to [claims 1 to 13 for preparing agents] **claim 1** for autologous immunotherapy.
29. A method for an immunotherapy which comprises *ex vivo* infection of immune cells with

a recombinant influenza virus according to [claims 1 to 13] **claim 1**, and introduction of the transduced cells into the patient.

30. A method for the induction of antibodies which comprises utilizing a recombinant influenza virus according to [claims 1 to 13] **claim 1** as an immunogen.

09/91465821502
518 Rec'd PCT/PTO 31 AUG 2001

"Express Mail" Label No. EL 867734861 US
Date of Deposit: August 31, 2001

Attorney Docket No.: Kreisler 1098-KGB
Client Ref. No.: 012142us/JH/ml

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS : GERD HOBOM, RAMON FLICK, ANETTE MENKE and MAYSA AZZEH
SERIAL NO. : TO BE ASSIGNED (International Application No.: PCT/EP00/01903)
FILED : HEREWITH
FOR : STABLE RECOMBINANT INFLUENZA VIRUSES FREE OF HELPER VIRUSES
ART UNIT : TO BE ASSIGNED
EXAMINER : TO BE ASSIGNED

August 31, 2001

Hon. Commissioner of Patents
Washington, D.C. 20231

STATEMENT REGARDING THE SEQUENCE LISTING

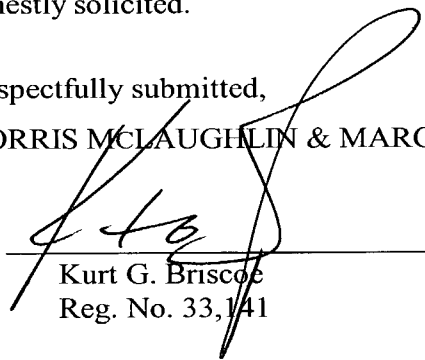
SIR:

Attached is a computer diskette containing a computer readable form of the paper Sequence Listing embedded in the specification. The undersigned hereby certifies that the paper Sequence Listing does not introduce new matter, and that the content of the paper Sequence Listing and the computer readable form copy is the same and includes no new matter.

Early and favorable action is earnestly solicited.

Respectfully submitted,
NORRIS MCLAUGHLIN & MARCUS, P.A.

By


Kurt G. Briscoe
Reg. No. 33,141

220 East 42nd Street - 30th Floor
New York, New York 10017
Phone: (212) 808-0700
Fax: (212) 808-0700



09914658 021502
09/914 658
Rec'd PCT/PTO 19 AUG 2002

~7048421
SEQUENCE LISTING

<110> Hobom, Gerd
Flick, Ramon
Menke, Anette
Azzeah, Maysa

<120> Stable Recombinant Influenza Viruses Free of Helper
Viruses

<130> Kreisler 1098

<140> US 09/914,658
<141> 2001-08-31

<140> PCT/EP00/01903
<141> 2000-03-03

<150> EP 99104519
<151> 1999-03-06

<160> 26

<170> Microsoft word XP

<210> 1
<211> 4930
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: pHL2969

<400> 1
cgtaggaagc ttctagaggg attggctgag acgaaaaaca tatgctagag ggattggctg 60
agacgaaaaa catatgctag agcggccgccc accgcggtgg agctccagct tttgttccct 120
ttagtgaggg ttaattgcgc gcaggcctag ctaggtaaag aaaaataccc ttgtttctac 180
taataacccg gcggcccaaa atgccgactc ggagcgaaag atatacctcc cccggggccg 240
ggaggtcgcg tcaccgacca cgccgcccgg ccaggcgacg cgcgacacgg acacctgtcc 300
ccaaaaacgc caccatcgca gccacacacg gagcgcccgg ggccctctgg tcaaccccag 360
gacacacgcg ggagcagcgc cgggcccggg acgccctccc ggccgcccgt gccacacgca 420
gggggcccgg ccgtgtctcc agagcgggag ccggaagcat tttcggccgg cccctcctac 480
gaccgggaca cacgagggac cgaaggccgg ccaggcgcca cctctcgggc cgcacgcgcg 540
ctcagggagc gctctccgac tccgcacggg gactcgccag aaaggatcgt gacctgcatt 600
aatgaatcag gggataacgc aggaagaagc atgtgagcaa aaggccagca aaaggccagg 660
aaccgtaaaa aggcgcggtt gctggcggtt ttccataggc tccgcccccc tgacgagcat 720
cacaaaaatc gacgctcaag tcagaggtgg cgaaacccga caggactata aagataccag 780
gcgtttcccc ctggaagctc cctcgtgcgc tctcctgttc cgaccctgcc gcttaccgga 840
tacgtgtccg cctttctccc ttccgggaagc gtggcgcttt ctcatagctc acgctgtagg 900
tatctcagtt cggtgtaggt cgttcgctcc aagctgggct gtgtgcacga acccccctgt 960
cagccccgac gctgcgcctt atccggtaac tatcgtcttg agtccaaccc ggtaagacac 1020
gacttatcgc cactggcagc agccactggt aacaggatta gcagagcgag gtatgtaggc 1080
gggtctacag agttcttgaa gtggtggcct aactacggct acactagaag gacagtattt 1140
ggtatctgcy ctctgctgaa gccagttacc ttcggaaaaa gagttggtag ctcttgatcc 1200
ggcaaaacaa ccaccgctgg tagcggtggt ttttttggtt gcaagcagca gattacgcgc 1260
agaaaaaaag gatctcaaga agatcctttg atcttttcta cggggtctga cgctcagtgg 1320
aacgaaaact caggttaagg gattttggct atgagattat caaaaaggat cttcacctag 1380
atccttttaa attaaaaatg aagtttttaa tcaattctaaa gtatatatga gtaaacttgg 1440
tctgacagtt accaatgctt aatcagtgag gcacctatct cagcgatctg tctatttcgt 1500
tcatccatag ttgcttgact ccccgctcgt tagataacta cgatacggga gggcttacca 1560
tctggcccca gtgctgcaat gataccgcga gaccacgctc caccggctcc agatttatca 1620

~7048421

```

gcaataaacc agccagccgg aagggccgag cgcagaagtg gtcctgcaac tttatccgcc 1680
tccatccagt ctattaattg ttgccgggaa gctagagtaa gtagttcgcc agttaatagt 1740
ttgcgcaacg ttgttgccat tgctacaggc atcgtggtgt cacgctcgtc gtttggtatg 1800
gcttcattca gctccggttc ccaacgatca aggcgagtta catgatcccc catgttgtgc 1860
aaaaaagcgg ttagctcctt cggtcctccg atcgttgta gaagtaagtt ggccgcagtg 1920
ttatcactca tggttatggc agcactgcat ctgtcatgcc atccgtaaga 1980
tgcttttctg tgactggtga gtactcaacc aagtcattct gagaatagtg tatgcggcga 2040
ccgagttgct cttgcccggc gtcaacacgg gataataccg cgccacatag cagaacttta 2100
aaagtgtcca tcattgaaa acgttcttcg gggcgaaaac tctcaaggat cttaccgctg 2160
ttgagatcca gttcagatga accactcgt gcacccaact gatcttcagc atcttttact 2220
ttcaccagcg tttctgggtg agcaaaaaa ggaaggcaaa atgccgcaaa aaaggggaata 2280
agggcgacac ggaaatgttg aatactcata ctcttccttt ttcaatatta ttgaagcatt 2340
tatcaggggt attgtctcat gagcggatac atatttgaat gtatttagaa aaataaacia 2400
aagagtttgt agaaacgcaa aaaggccatc cgctcagatg gccttctgct taatttgatg 2460
cctggcagtt tatggcgggc gtcttgccc ggcactccg ggccgttgct tcgcaacggt 2520
caaatccgct cccggcggat ttgtcctact caggagagcg ttcaccgaca aacaacagat 2580
aaaacgaaag gccagtcct tcgactgagc ctttcgtttt atttgatgcc tggcagttcc 2640
ctactctcgc acctagagac cccacactac acggcgcttc acggcgcttc acttctgagt 2700
tcggcatggg gtcaggtggg accaccgcgc tactgccgcc aggcacaaatc tgttttatca 2760
gaccgcttct gcgttctgat ttaatctgta tcaggctgaa aatcttctct catccgcaa 2820
aacagaagct agcggccgat ccccaaaaaa aaaaaaaaaa aaaaaaaaaa gagtccagag 2880
tgcccccgcc gttccgcgcc gggggggggg gggggggggg acactttcgg acatctggct 2940
gacctccagc atcgggggaa aaaaaaaaaa caaagtctcg cccggagtag cgttgagcaa 3000
ccgaagtggg gggggagtag aaacagggta gataatcact cactgacgta cgttgagcaa 3060
ctgactgaaa tgccttgagc aactgactga aatgcctgac gtcttttagc aaagcaggt 3120
agataatcac tcagtggagc acatccacga ttggctgaga gtacaaaaat gaacactcaa 3180
attgtaccag ggattggctg agacgaaaaa catattgtag gtacaaaaat ttgtcttggg 3240
atcctggttt tcgcccttgc ggcagtcata cccacaaatg cagacaaaat ctgagagagg 3300
catcatgctg tatcaaatgg caccaaagta aacacactca ctgagagagg agtagaagtt 3360
gtcaatgcac gttccgcgcc cggaacgaca agcagtgact aaatttgctc aaaagggaag 3420
agaaccactg atcttgacca atgcggactg ttagggacca ttaccggacc acctcaatgc 3480
gaccaatttc tagaattttc agctgatcta ataactgaga gacgagaagg aaatgatgtt 3540
tgttaccggg ggaagtgtgt taatgaagag gcattgagac aaatcctcag aggatcaggt 3600
gggattgaca aagaaacaat gggattcaca tatagtggaa taaggaccaa cggacaact 3660
agtgcattga gaagatcagg gcttcttccc tttgtagggt tggagtggct cctgtcaaat 3720
acagacaatg cttctttccc tctggggaat ccaccattca ggatcaacca cccaacagac 3780
gctctgatag ataaactgat aacagtcggg agttccaaat atcatcaatc ttttgtgccg 3840
gggagtggaa cacgaccgca gataaatggc cgttcaggac ggattgattt tcattgggtg 3900
agtcaggaa ccaatgatac agttactttt agtttcaatg gggctttcat agctccaaat 4020
atcttggatc cgtgccagct tcttgagggg aaagtccatg gggatccaga gcgatgtgca ggttgatgct 4080
cgtgccagct aattgcgaag ggaatgcta ccacagtggg gggagataaa caagcagatt gccttttcaa 4140
aacataaata gcagagcagt tggcaaatgc ccaagatatg taaaacagga aagtttatta 4200
ttggcaactg ggaatgaaga cgttcccgaa ccttccaaaa aaaggaaaaa aagaggcctg 4260
tttggtgcta tagcaggggt tattgaaaat ggttggaag gtctggtcga cgggtggtac 4320
ggtttcaggc atcagaatgc acaaggagaa ggaactgcag cagactacaa aagcacccaa 4380
tcggcaattg atcagataac cgaaaagtta aatagactca ttaagaaaac caaccagcaa 4440
tttgagctaa tagataatga attcactgaa gtggaaaagc agattggcaa tttaattaac 4500
tggaccaaa gactccatcac agaagtatgg tcttacaatg ctgaacttct tgtggcaatg 4560
gaaaaccagc acactattga tttggctgat tcagagatga acaagctgta tgagcgagtg 4620
agggaaacaat taagggaata tgctgaagag gatggcactg gttgctttga aatttttcat 4680
aaatgtgacg atgattgtat ggctagtata aggaacaata cttatgatca cagcaaatat 4740
agagaagaag cgatgcaaaa tagaatacaa attgaccag tcaaattgag tagtggctac 4800
aaagatgtga tactttggtt tagcttcggg gcatcatgct ttttgcttct tgccattgca 4860
atgggccttg ttttcatatg tgtgaagaac ggaacatgc ggtgcactat atgcatttaa 4920
agcttgcatt 4930

```

<210> 2

<211> 5811

<212> DNA

<213> Artificial sequence

~7048421

<220>

<223> Description of Artificial Sequence: pAM403

<400> 2

```

aattcctttg cctaatttaa atgaggactt aacctgtgga aatattttga tgtgggaagc 60
tgttactgtt aaaactgagg ttattggggg aactgctatg ttaaacttgc attcagggac 120
acaaaaaact catgaaaatg gtgctggaaa acccattcaa gggcctaaatt ttcatttttt 180
tgctgttggg ggggaacctt tggagctgca ggggtgtgta gcaaactaca ggaccaaata 240
tcctgctcaa actgtaaccc caaaaaatgc tacagttgac agtcagcaga tgaacactga 300
ccacaaggct gttttggata aggataatgc ttatccagtg gagtgctggg ttcctgatcc 360
aagtaaaaat gaaaacacta gatatttttg aacctacaca ggtggggaaa atgtgcctcc 420
tgttttgcac attactaaca cagcaaccac agtgcttctt gatgagcagg gtgttgggcc 480
cttgtgcaaa gctgacagct tgtatgtttc tgcgttgac atttgtgggc tgtttacca 540
cacttctgga acacagcagt ggaaggagct tccagatat ttaaaatta cccttagaaa 600
gcggtctgtg aaaaaccctt acccaatttc cttttgtta agtgacctaa ttaacaggag 660
gacacagagg gtggatgggc agcctatgat tggatgtcc tctcaagtag aggaggttag 720
ggtttatgag gacacagagg agcttcctgg ggaatccagac atgataagat acattgatga 780
gtttggacaa caattctgag gaataaatgc tttatttggg aaatttgtga 840
tgctattgct ttatttgtaa ccattataag ctgcaataaa caagttaaca acaacaattg 900
cattcatttt atgtttcagg ttcaggggga ggtgtgggag gttttttaa gcaagtaaaa 960
ccttcacaaa tgtggtatgg ctgattatga tctctagtca aggcactata catcaaatat 1020
tccttattaa cccctttaca aattaaaaag caattctgaa aagaaggtac acaatttttg agcatagtta 1080
ttaatagcag acactctatg cctgtgtgga gtaagaaaaa acagtatggt acagtattgt 1140
ctgttatgcc tacttataaa gggtacagaa tatttttcca taattttctt gtatagcagt 1200
gcagcttttt cctttgtggt gtaaatagca aagcaagcaa gagttctatt actaaacaca 1260
gcatgactca aaaaacttag caattctgaa ggaagtcctt tggggctctt cctcatcatc actagatggc 1320
ttcttttttg gaggagtaga atgttgagag tcagcagtag cctcatcatc actagatggc 1380
atcttctctg agcaaaacag gtttctctca ttaaaggcat tccaccactg ctccattca 1440
tcagttccat aggttggaa ctaaaatata caaacaatta gaatcagtag tttaacacat 1500
tatacctta aaaattttat atttacctta gagctttaaa tctctgtagg tagtttgtcc 1560
aattatgtca caccacagaa gtaagggttc ttcacaagaa tccgggacca aagcggccat 1620
cgtgcctccc cactcctgca gttcgggggc atggatgcgc ggatagccgc tgctggtttc 1680
ctggatgccg acggatttgc actgcccgtg gaactccgcg aggtcgtcca gcctcaggca 1740
gcagctgaac caactgcga ggggatcgag cccgggggtg gcgaagaact ccagcatgag 1800
atccccgcgc tggaggatca tccagccggc gtcgggaaa acgattccga agcccaacct 1860
ttcatagaag gcggcgggtg aatcgaaatc tcgtgatggc aggttgggag tgcgttggtc 1920
ggtcatttct atgaattcga gctcgggtacc cggggatcct ctgagggcat ttcagtttct 1980
tcctcacgga ctcatcagag tgctcgaatt cgaacccag agtcccgtc agaagaactc 2040
gtcaagaagg cgatagaagg cgatgcgctg cgaatccgga gcggcgatac cgtaaaagca 2100
gaggaagcgg tcagcccatt cgccgccaag ctcttcagca atatcacggg tagccaacgc 2160
tatgtcctga tagcgggtcg ccacaccag ccggccacag tcgatgaatc cagaaaagcg 2220
gccattttcc accatgatat tcggcaagca ggcacgcca tgggtcacga cgagatcctc 2280
gccgtcgggc atgcgcgctt tgagcctggc gaacagttcg gctggcgca gcccctgatg 2340
ctcttcgtcc agatcatcct gatcgacaag accggcttcc atccgagtag gtgctcgctc 2400
gatgcgatgt ttcgcttggg ggtcgaatgg gcaggtagcc ggatcaagcg tatgcagccg 2460
ccgattgca tcagccatga tggatacttt ctggcagga gcaaggtgag atgacaggag 2520
atcctgcccc ggcacttcgc ccaatagcag ccagtcctt cccgcttcag tgacaacgct 2580
gagcacagct gcgcaaggaa cgcccgctcg ggccagccac gatagccgcg ctgcctcgct 2640
ctgcagttca ttcagggcac cggacaggtc ggtcttgaca aaaagaaccg ggcgcccctg 2700
cgctgacagc cggaacacgg cggcatcaga gcagccgatt gtctgttgtg cccagtcata 2760
gccgaatagc ctctccaccc aagcggccgg agaacttgcg tgcaatccat cttgttcaat 2820
catgcgaaac gatcctcatc ctgtctcttg atcagatctt gatcccctgc gccatcagat 2880
ccttggcggc aagaaagcca tccagtttac tttgcagggc ttcccaacct taccagaggg 2940
cgccccagct ggcaattccg gttcgcttgc tgtccataaa accgcccagt ctgctatctg 3000
ccatgtaagc ccactgcaag ctactgctt tctcttttgc cttgcgtttt cccttgtcca 3060
gatagcccag tagctgacat tcatccgggg tctagccagt ttctgaggac tggctttcta 3120
cgtgttccgc ttcctttagc agcccttgcg ccttgagtgc ttgcccagc gtgaagcttt 3180
ttgcaaaagc ctaggcctcc aaaaaagcct cctcactact tctggaatag ctgagaggcc 3240
gaggcgccct cggcctctgc ataaataaaa aaattagtc agccatgggg cggagaatgg 3300
gcggaactgg gcggagttag gggcgggatg gggcggggac tatggttctg 3360
gactaattga gatgcatgct ttgcatactt ctgcctgctg gggagcctgg ggactttcca 3420
cacctggttg ctgactaatt gagatgcatg ctttgcatac ttctgcctgc tggggagcct 3480
ggggactttc cacaccctaa ctgacacaca ttccacagct gcctcgcgcg tttcggtagt 3540

```

~7048421

gacgggtgaaa	acctctgaca	catgcagctc	ccggagacgg	tcacagcttg	tctgtaagcg	3600
gatgccggga	gcagacaagc	ccgtcagggc	gcgtcagcgg	gtgttggcgg	gtgtcggggc	3660
gcagccatga	cccagtcacg	tagcgatagc	ggagtgtata	ctggcttaac	tatgcggcat	3720
cagagcagat	tgtactgaga	gtgcaccata	tgcggtgtga	aataccgcac	agatgcgtaa	3780
ggagaaaata	ccgcatcagg	cgctcttccg	cttccctcgt	cactgactcg	ctgcgctcgg	3840
tcgttcgggt	gcggcgagcg	gtatcagctc	actcaaaggc	ggtaatacgg	ttatccacag	3900
aatcagggga	taacgcagga	aagaacatgt	gagcaaaagg	ccagcaaaag	gccaggaacc	3960
gtaaaaaggc	cgcggttgctg	gcgtttttcc	ataggctccg	ccccctgac	gagcatcaca	4020
aaaatcgacg	ctcaagtcag	aggtggcgaa	acccgacagg	actataaaga	taccaggcgt	4080
ttccccctgg	aagctccctc	gtgcgctctc	ctgttccgac	cctgccgctt	accggatacc	4140
tgtccgcctt	tctcccttcg	ggaagcgtag	cgcttctca	tagctcacgc	tgtaggatct	4200
tcagttcggg	gtaggtcggt	cgctccaagc	tgggctgtgt	gcacgaaccc	cccgttcagc	4260
ccgacccttg	cgccttatcc	ggtaactatc	gtcttgagtc	caacccggta	agacacgact	4320
tatcgccact	ggcagcagcc	actggtaaca	ggattacgag	agcgaaggat	gtaggcggtg	4380
ctacagagtt	cttgaagtg	tggcctaact	acggctacac	tagaaggaca	gtatttggtg	4440
tctgcgctct	gctgaagcca	gttaccttcg	gaaaaagagt	tggtagctct	tgatccggca	4500
aaacaaaccac	cgtggtagc	ggtggttttt	ttgtttgcaa	gcagcagatt	acgcgcagaa	4560
aaaaaggatc	ctcagaagat	cttttgatct	ctttgacggg	gtctgacgct	cagtggaaacg	4620
aaaactcacg	ttaagggatt	ttgggtcatga	gattatcaaa	aaggatcttc	acctagatcc	4680
ttttaaatta	aaaatgaagt	tttaaataca	tctaaagtat	atatgagtaa	acttgggtctg	4740
acagttacca	atgcttaatc	agtgaggcac	ctatctcagc	gatctgtcta	tttcgttcat	4800
ccatagttgc	ctgactcccc	gtcgtgtaga	taactacgat	acgggagggc	ttaccatctg	4860
gccccagtg	tgcaatgata	ccgcgagacc	cacgctcacc	ggctccagat	ttatcagcaa	4920
taaaccagcc	agccggaagg	gccgagcgca	gaagtgggtc	tgcaacttta	tccgcctcca	4980
tccagtcctat	taattgttgc	cgggaagcta	gagtaagtag	ttcgccagtt	aatagtttgc	5040
gcaacgttgt	tgccattgct	gcaggcatcg	tgggtgtcag	ctcgtcggtt	ggtagggctt	5100
cattcagctc	cggttcccaa	cgatcaaggc	gagttacatg	atcccccatg	ttgtgcaaaa	5160
aagcggttag	ctccttcggt	ctccgatcg	ttgtcagaag	taagttggcc	gcagtgttat	5220
cactcatggt	tatggcagca	ctgcataatt	ctcttactgt	catgccatcc	gtaagatgct	5280
tttctgtgac	tggtgagtac	tcaaccaagt	catcttgaga	atagtgtatg	cggcgaccga	5340
gttgctcttg	cccggcgctca	acacgggata	ataccgcgcc	acatagcaga	actttaaaag	5400
tgctcatcat	tggaaaacgt	tcttcggggc	gaaaactctc	aaggatctta	ccgctgttga	5460
gatccagttc	gatgtaaccc	actcgtgcac	ccaactgatc	ttcagcatct	tttactttca	5520
ccagcgtttc	tgggtgagca	aaaacaggaa	ggcaaaaatgc	cgcaaaaaag	ggaataaggg	5580
cgacacggaa	atgttgaaata	ctcatactct	tcctttttca	atattattga	agcatttatc	5640
aggggtattg	tctcatgagc	ggatacatat	ttgaatgtat	ttagaaaaat	aaacaaatag	5700
gggttcgcgcg	cacatttccc	cgaaaagtgc	cacctgacgt	ctaagaaacc	attattatca	5760
tgacattaac	ctataaaaat	aggcgtatca	cgaggccctt	tcgtcttcaa	g	5811

<210> 3

<211> 2005

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: vHM41

<400> 3

agtagaaaca	aggggtatttt	tctttacctta	gctaggcctg	cgcgcaatta	accctcacta	60
aagggaaaca	aagcttggagc	tccaccgcgg	tggcgccgcg	tctagcatat	gtttttcgtc	120
tcagccaatc	cctctagcat	atgttttttcg	tctcagccaa	tccctctaga	agcttcgtac	180
gcatgcaagc	tttaaattgca	tatagtgac	cgcatgtttc	cgttcttcac	acatatgaaa	240
acaaggccca	ttgcaatggc	aagaagcaaa	aagcatgatg	ccccgaagct	aaaccaaagt	300
atcacatctt	tgtagccact	actcaatttg	actgggtcaa	tttgatttct	attttgcatc	360
gcttcttctc	tgtatttgct	gtgatcataa	gtattgttcc	ttatactagc	catacaatca	420
tcgtcacatt	tatgaaaaat	ttcaaagcaa	ccagtgccat	cctcttcagc	attttccctt	480
aattgtttcc	tcactcgctc	atacagcttg	ttcatctctg	aatcagccaa	atcaatagtg	540
tgctgggttt	ccattggcac	aagaagttaa	gcattgtaag	accatacttc	tgtgatggag	600
tctttgggtcc	agttaattaa	attgccaatc	tgtttttcca	cttcagtgaa	ttcatttatc	660
attagctcaa	attgctgggt	ggttttctta	atgagtctat	ttaaactttc	ggttatctga	720
tcaattgccg	attgggtgct	ttttagtctt	gctgcagttc	cttctccttg	tgcatctctga	780
tgcttgaaac	cgtaccaccc	gtcgaccaga	ccttcccaac	cattttcaat	aaaccctgct	840

~7048421

```

atagcaccaa acaggcctct ttttttcctt tttttggaag gttcgggaac gttcttcac 900
ccagttgcca ataataaact ttctgtttt acatatcttg ggcatttgcc aactgctctg 960
ctatttatgt tttgaaaagg caatctgctt gttatagtcc ctccactgtg gtagcattcc 1020
ccttcgcaat tagcatcaac ctgcacatcg ctctggatcc ccatggactt tcccctcaag 1080
aagctggcac gatttggagc tatgaaagcc ccattgaaac taaaagtaac tgtatcattg 1140
ggatccaaga tcaaccaatg aaaatcaatc cgtccggacc ggccatttat ctgcggtcgt 1200
gttcctggac tcggcacaac agattgatga tatttggaaac tcccgaactgt tatcagttta 1260
tttccactcc catatagttt ggtctgttct gtggttgatc ctgaatggtg gattccccag 1320
actatcagag ctgattctct ccctgtgttt ttgtatgatt ttgtcatttg tgggaaagaa 1380
gcattgtctg tatttgacag gagccactcc atttctgcat agaatagaaga cctgatctt 1440
ctacatgcac tagttgttcc gttggtcctt attccactat atgtgaatcc cattgtttct 1500
ttgtcaatcc cacctgatcc tctgaggatt tgtcgcgaat cctcttcatt aacaaacttc 1560
cccgggtaac aaacatcatt tccttctcgt ctctcgatta ttagatcagc tgaaaattct 1620
agaaattggt cgcattgagg ttgtccggtt atgttcccta acagtcgcga ttggccaaga 1680
tcagtgggtc ttttcccttt tgagcaaatt ttggggatgt ttgtccgctc cactgtttcc 1740
gttgcatgga caacttctac tcctctctca gtgagtgtgt ttactttggt gccatttgat 1800
acagcatgat gtccaagaca aattttgtct gcatttgtgg ggatgactgc cgcaagggcg 1860
aaaaccagga tttgagtgtt cattttggtt cctacaatat gtttttcgtc tcagccaatc 1920
cctggtacaa tatgtttttc gtctcagcca atcctggtac gatgtggatg tcactcagt 1980
agtgtattac taccctgctt ttgct                                     2005

```

<210> 4

<211> 1146

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: vHM81

<400> 4

```

agtagaaaca agggatattt tctttaccta gctaggcctg cgcgcaatta accctcacta 60
aagggaacaa aagctggagc tccaccgcgg tggcggccgc tctagcatat gtttttcgtc 120
tcagccaatc cctctagcat atgtttttcg tctcagccaa tccctctaga agcttcgtac 180
gcatgcttaa ataagctgaa acgagaaaagt tcttatctct tgctccactt caagcggtag 240
ttgtaaggct tgcataaatg ttatttgttc aaaactattc tctgttatct tcaatctatg 300
tctcacttct tcaattaacc atcttatttc ttcaaatttc tgactcaatt gttctcgcca 360
ttttccggtt ctgctttgga gggagtggag gtccccatt ctcattactg cttctccaag 420
cgaatctctg tatagtttca gagactcgaa ctgtgttatc attccattca agtcctccga 480
tgaggacccc aattgcattt ttgacatcct catcagtatg tcctggaaga gaaggcaatg 540
gtgaaatttc gccgacaatt gctccctcat cggttaaagc ccttaatagt atgagagttt 600
ccagccgatc gaaaatcaca ctgaagtttg ctttcagtat gatgttcttc ccatgatcgc 660
cctgggtccat tctgatgcaa agggagcctg ccactttctg tttgggcatg agcatgaacc 720
agtcccttga catctcttca agagtcatgt cagttaggta gcgtgtagca ggtacagagg 780
caatgggtcat tttaagtgcc tcatcgatt cgctctccag aatccgctcc actatctgct 840
ttccaacacg agtagctgtg tcgatgtcca gaccaagagt gctgcctctt cccctcaggg 900
acttctgatc tcggcgaagt cggccaagga atggggcatc acccatttct tggctgcaa 960
atcgtttgcg gacatgccaa agaaagcagt ctacctgaaa gcttgacaca gtgttggaat 1020
ccattatggt acctacaata tgtttttcgt ctacagcaat ccctggtaca atatgtttt 1080
cgtctcagcc aatcctggta cgatgtggat gtcactcagt gagtgattat ctaccctgct 1140
tttgct

```

<210> 5

<211> 5860

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: pAM424

<400> 5

```

catcgattgg ctgactgatg agtccgtgag gacgaaacga aaaacatatt gtagagctcg 60

```

~7048421

aattcatcga	aatgaccgac	caagcgagcg	ccaacctgcc	atcacgagat	ttcgattcca	120
ccgccgcctt	ctatgaaagg	ttgggcttcg	gaatcgTTTT	ccgggacgcc	ggctggatga	180
tcctccagcg	cggggatctc	atgctggagt	tcttcgcccc	ccccgggctc	gatccccctg	240
cgagttgggt	cagctgctgc	ctgaggctgg	acgacctcgc	ggagttctac	cggcagtgca	300
aatccgctcg	catccaggaa	accagcagcg	gctatccgcg	catccatgcc	cccgaactgc	360
aggagtgggg	aggcacgatg	gccgctttgg	tcccgatctt	ttgtgaagga	accttacttc	420
tgtgggtgga	cataattgga	caaactacct	acagagattt	aaagctctaa	ggtaaatata	480
aaatTTTTaa	gtgtataatg	tgTTaaacta	ctgatttctaa	ttgtttgtgt	atttttagatt	540
ccaacctatg	gaactgatga	atgggagcag	tggtggaatg	cctttaatga	ggaaaacctg	600
TTTTgtctag	aagaaatgcc	atctagtgat	gatgaggcta	ctgctgactc	tcaacattct	660
actcctccaa	aaaagaagag	aaaggtagaa	gaccccaagg	actttccttc	agaattgcta	720
agTTTTttga	gtcatgctgt	gttttagtaat	agaactcttg	cttgctttgc	tatttacacc	780
caaaaggaaa	aagctgcact	gctatacaag	aaaattatgg	aaaaatattc	tgtaaccttt	840
ataagttagc	ataacagtta	taatcataac	taactgtttt	ttcttactcc	acacaggcat	900
agagtgtctg	ctattaataa	ctatgctcaa	aaattgtgta	ccttttagctt	tttaatttgt	960
aaaggggtta	ataaggaata	tttgatgtat	agtgccttga	ctagagatca	taatcagcca	1020
taccacattt	gtagaggttt	tacttgcttt	aaaaaacctc	ccacacctcc	ccctgaacct	1080
gaaacataaa	atgaatgcaa	ttgttgtgtt	taacttgttt	attgcagctt	ataatgggta	1140
caaataaagc	aatagcatca	caaatttcac	aaataaagca	tttttttcac	tgcatttctag	1200
ttgtggTTTT	tcCAAactca	tcaatgtatc	ttatcatgtc	tggatcccca	ggaagctcct	1260
ctgtgtcttc	ataaaacctc	acctcctcta	cttgagagga	cattccaatc	ataggctgcc	1320
catccacctc	ctgtgtcctc	ctgttaatta	ggtcacttaa	caaaaaggaa	attgggtagg	1380
ggTTTTttcac	agaccgcttt	ctaagggtaa	ttttaaaata	tctgggaagt	cccttcactt	1440
gctgtgttcc	agaagtgttg	gtaaacagcc	cacaaatgtc	aacagcagaa	acatacaagc	1500
tgctcagctt	gcacaagggc	ccaacaccct	gctcatcaag	aagcactgtg	gttgctgtgt	1560
tagtaatgtg	caaaacagga	ggcacatttt	ccccacctgt	gtaggttcca	aaatatctag	1620
tgTTTTcatt	tttacttgga	tcaggaacct	agcactccac	tggataagca	ttatccttat	1680
ccaaaacagc	cttggtgtca	gtgttcatct	gctgactgtc	aactgtagca	TTTTttgggg	1740
ttacagtttg	agcaggatat	ttggtcctgt	agtttgctaa	cacaccctgc	agctccaaag	1800
gttccccacc	aacagcaaaa	aaatgaaaa	ttgacccttg	aatgggtttt	ccagcaccat	1860
tttcatgagt	TTTTgtgtc	cctgaatgca	agtttaacat	agcagttacc	ccaataacct	1920
cagttttaac	agtaacagct	tcccacatca	aaatatTTcc	acaggttaag	tcctcattta	1980
aattaggcaa	aggaattctt	gaagacgaaa	gggcctcgtg	atacgcctat	TTTTataggt	2040
taatgtcatg	ataataatgg	tttcttagac	gtcagggtgg	acttttcggg	gaaatgtgcg	2100
cggaacccct	atttgTTTT	ttttctaaat	acattcaaat	atgtatccgc	tcatgagaca	2160
ataaccctga	taaatgcttc	aataatatTg	aaaaagggaag	agtatgagta	ttcaacattt	2220
ccgtgtcgcc	cttattccct	TTTTtgcggc	atTTtgctt	cctgtTTTTg	ctcaccacga	2280
aacgttggtg	aaagtaaaag	atgctgaaga	tcagttgggt	gcacgagtg	gttacatcga	2340
actggatctc	aacagcggtg	agatccttga	gagttttcgc	cccgaagaac	gttttccaat	2400
gatgagcact	tttaaagtTc	tgctatgtgg	cgcggtatta	tcccgtgttg	acgccgggca	2460
agagcaactc	ggtcgcccga	tacactattc	tcagaatgac	ttggttgagt	actcaccagt	2520
cacagaaaag	catcttacgg	atggcatgac	agtaagagaa	ttatgcagtg	ctgccataac	2580
catgagtgat	aacactgcgg	ccaacttact	tctaacaacg	atcggaggac	cgaaggagct	2640
aaccgctttt	ttgcacaaca	tgggggatca	tgtaactcgc	cttgatcggt	gggaaccgga	2700
gctgaatgaa	gccataccaa	acgacgagcg	tgacaccacg	atgcctgcag	caatggcaac	2760
aacgttgcg	aaactattaa	ctggcgaaact	acttactcta	gcttcccggc	aacaattaat	2820
agactggatg	gaggcgagata	aagttgcagg	accacttctg	cgctcggccc	ttccggctgg	2880
ctggtttatt	gctgataaat	ctggagccgg	tgagcgtggg	tctcgcggta	tcattgcagc	2940
actggggcca	gatggtaagc	cctcccgtat	cgtagttatc	tacacgacgg	ggagtcaggc	3000
aactatggat	gaacgaaata	gacagatcgc	tgagataggt	gcctcactga	ttaagcattg	3060
gtaactgtca	gaccaagttt	actcatatat	actttagatt	gatttaaaac	ttcattttta	3120
atttaaaagg	atctaggtga	agatcctttt	tgataatctc	atgaccaaaa	tcccttaacg	3180
tgagttttcg	ttccactgag	cgtcagaccc	cgtagaaaag	atcaaaggat	cttcttgaga	3240
tcctTTTTtt	ctgcgcgtaa	tctgtgctt	gcaaacaaaa	aaaccaccgc	taccagcggg	3300
ggtttgTTTT	cgggatcaag	agctaccaag	tctttttccg	aaggtaactg	gcttcagcag	3360
agcgcagata	ccaaatactg	tccttctagt	ttaggccacc	ttaccagtg	acttcaagaa	3420
ctctgtagca	ccgcctacat	acctcgctct	gctaactcct	tagttaccgg	ctgctgccag	3480
tggcgataag	tcgtgtctta	ccgggttggg	ctcaagacga	ttggagcgaa	ataaggcgca	3540
gcggtcgggc	tgaacggggg	gttcgtgcac	acagcccagc	acgcttccc	cgacctacac	3600
cgaactgaga	tacctacagc	gtgagctatg	agaaacgcc	gagcgcacga	aagggagaaa	3660
ggcggacagg	tatccggtaa	gcggcagggg	cggaacagga	cgccacctct	gggagcttcc	3720
agggggaaac	gcctggtatc	tttatagTcc	tgTcgggttt	gacttgagcg	gacttgagcg	3780
tcgatttttg	tgatgctcgt	cagggggg	gagcctatgg	gcaacgcggc		3840

~7048421

```

cttttttacgg ttcctggcct tttgctggcc ttttgctcac atgtttctttc ctgctgtatc 3900
ccctgattct gtggataacc gtattaccgc ctttgagtga gctgataccg ctcgccgcag 3960
ccgaacgacc gagcgagcg agtcagtgag cgaggaagcg gaagagcgcc tgatgcggta 4020
ttttctcctt acgcatctgt gcggtatttc acaccgcata tgggtgcactc tcagtacaat 4080
ctgctctgat gccgcatagt taagccagta taccctccgc tatcgctacg tgactgggtc 4140
atggctgcgc cccgacaccc gccaacaccc gctgacgcgc cctgacgggc ttgtctgtc 4200
ccggcatccg cttacagaca agctgtgacc gtctccggga gctgcatgtg tcagaggttt 4260
tcaccgtcat caccgaaacg cgcgaggcag ctgtggaatg tgtgtcagtt aggggtgtga 4320
aagtccccag gctccccagc aggcagaagt atgcaaagca tgcattctca ttagtcagca 4380
accagggtgtg gaaagtcccc aggtccccca gcaggcagaa gtatgcaaag catgcatctc 4440
aattagtcag caaccatagt cccgccccta tcccgcctt aactccgccc 4500
agttccgccc atttctccgc ccatggctga ctaatttttt ttattttatg agaggccgag 4560
gccgcctcgg cctctgagct attccagaag tagtgaggag gcttttttgg aggcctaagg 4620
ttttgcaaaa agcttcacgc tgccgcaagc actcagggcg caagggctgc taaaggaagc 4680
ggaaacgta gaaagccagt ccgcagaaac ggtgtgacc ccgatgaat gtcagctact 4740
gggctatctg gacaagggaa aacgcaagcg caaagagaaa gcaggtagct tgcagtggg 4800
ttacatggcg atagctagac tgggcgggtt tatggacagc aagcgaaccg gaattgccag 4860
ctggggcgcc ctctggttaag gtgggaagc cctgcaaagt aaactggatg gctttcttgc 4920
cgccatcgag ctgatggcgc aggggatcaa gatctgatca agagacagga tgaggatcgt 4980
ttcgcagatg tgaacaagat ggattgcacg caggttcttc ggccgcttgg gtggagaggc 5040
tattcggcta tgactgggca caacagacaa tcggctgctc tgatgccgcc gtgtccggc 5100
tgtcagcgca ggggcgcccc gtcttttttg tcaagaccga cctgtccggg gccctgaatg 5160
aactgcagga cggagcagc cggctatcgt gactggccac gacgggcgtt ccttgccgag 5220
ctgtgctcga cgttgtcact gaagcgggaa gggactggct gctattgggc gaagtgccgg 5280
ggcaggatct cctgtcatct caccttgctc ctgcccagaa agtatccatc atggctgatg 5340
caatgcggcg gctgcatacg cttgatccgg ctacctgccc attcgaccac caagcgaac 5400
atcgcatcga gcgagcacgt actcgatgg agcccggtct tgcgatcag gatgatctgg 5460
acgaagagca tcaggggctc gcgcagccg aactgttcgc caggctcaag gcgcgcatgc 5520
ccgacggcga ggatctcgtc gtgacccatg gcgatgcctg cttgccgaat atcatgggtg 5580
aaaatggccg cttttctgga ttcatcgact gtggccggct ggggtgtggc gaccgctatc 5640
aggacatagg gttggctacc cgtgatattg ctgaagagct tggcggcgaa tgggctgacc 5700
gcttcctcgt gctttacggg atcgccgctc ccgattcgca gcgcatcgcc ttctatcgcc 5760
ttcttgacga gttcttctga gcgggactct ggggttcgaa tcctaccagg gattggctga 5820
ctgatgagtc cgtgaggacg aaacgaaaaa catatggtac 5860

```

<210> 6

<211> 4610

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: pHL2507

<400> 6

```

gaggcatttc agtcagttgc tcaagggtacc aaaatgaaca ctcaaattcct ggttttcgcc 60
cttgccgcag tcatccccac aaatgcagac aaaatttgtc ttggacatca tgctgtatca 120
aatggcacca aagtaaacac actcactgag agaggagtag aagttgtcaa tgcaacggaa 180
acagtggagc ggacaaacat ccccaaaatt tgctcaaaag ggaaaagaac cactgatctt 240
ggccaatgcg gactgttagg gaccattacc ggaccacctc aatgcgacca atttctagaa 300
ttttcagctg atctaataat cgagagacga gaaggaaatg atgtttgtta cccggggaag 360
tttggttaat aagaggcatt gcgacaaatc ctgagaggat cagggtgggag tgacaaagaa 420
acaatgggat tcacatatag tgaataaagg accaacggaa caactagtgc atgtagaaga 480
tcagggtctt cattctatgc agaattggag tggctcctgt caaatacaga caatgcttct 540
ttcccacaaa tgacaaaatc atacaaaaac acagggagag aatcagctct gatagtctgg 600
ggaatccacc attcaggatc aaccaccgaa cagaccaaac tatatgggag tggaaataaa 660
ctgataacag tcgggagttc caaatatcat caatcttttg tgccgagtcc aggaacacga 720
ccgcagataa atggccgggt cggacggatt gattttcatt ggttgatctt ggatcccaat 780
gatacagtta ctttttagttt caatggggct ttcatagctc caaatcgtgc cagcttcttg 840
aggggaaagt ccatggggat ccagagcgat gtgcaggttg atgctaattg cgaaggggaa 900
tgctaccaca gtggagggac tataacaagc agattgcctt ttcaaacat aaatagcaga 960
gcagttggca aatgcccag atagttaaaa caggaaagtt tattattggc aactgggatg 1020

```

~7048421

aagaacgttc	ccgaaccttc	caaaaaaagg	aaaaaaaagag	gcctgtttgg	tgctatagca	1080
gggtttattg	aaaatggttg	ggaaggtctg	gtcgacgggt	ggtacggttt	caggcatcag	1140
aatgcacaag	gagaaggaac	tgcagcagac	tacaaaagca	cccaatcggc	aattgatcag	1200
ataaccggaa	agttaaatag	actcattaag	aaaaccaacc	agcaatttga	gctaatagat	1260
aatgaattca	ctgaagtgga	aaagcagatt	ggcaatttaa	ttaactggac	caaagactcc	1320
atcacagaag	tatggtctta	caatgctgaa	cttcttgtgg	caatggaaaa	ccagcacact	1380
attgattttg	ctgattcaga	gatgaacaag	ctgtatgagc	gagtgaggaa	acaattaagg	1440
gaaaatgctg	aagaggatgg	cactggttgc	tttgaaattt	ttcataaatg	tgacgatgat	1500
tgtatggcta	gtataaggaa	caatacttat	gatcacagca	aatacacaga	agaagcgatg	1560
caaaatagaa	tacaaattga	cccagtcaaa	ttgagttagt	gctacaaaga	tgtgatactt	1620
tggtttagct	tcggggcatc	atgctttttg	cttcttgcca	ttgcaatggg	ccttggtttc	1680
atatgtgtga	agaacggaaa	catgcggtgc	actatttgta	tatagggttg	gaaaaaaaca	1740
ccccttggtt	ctactccccc	ccaacttcgg	aggtcgacca	gtactccggg	cgaacttttg	1800
tttttttttt	ttcccccgat	gctggagggt	gactgagagt	ccgaaagtgt	ccccccccc	1860
ccccccccc	ggcgcggaac	ggcggggcca	ctctggactc	tttttttttt	tttttttttt	1920
ttttttgggg	atcgggcgct	agcttctgtt	ttggcggtat	agagaagatt	ttcagcctga	1980
tacagattaa	atcagaacgc	agaagcggtc	tgataaaaaca	gaatttgcct	ggcggcagta	2040
cggcgggtgg	cccactgac	cccatgcccga	actcagaagt	gaaacgccgt	agcgccgatg	2100
gtagtgtggg	gtctcccat	gcgagagtag	ggaactgccca	ggcatcaaat	aaaacgaaag	2160
gctcagtcga	aagactgggc	ctttcggttt	atctgttgtt	tgctcggtgaa	cgctctcctg	2220
agtaggacaa	atccgcccgg	agcggatttg	aacgttgcca	agcaacggcc	cggagggtgg	2280
cggcgaggac	gcccgccata	caatcaaatga	ctcgaattga	agcagaaggc	catcctgacg	2340
gatggccttt	ttgcgtttct	acaaactctt	ttgtttattt	ttctaaatac	attcaaatat	2400
gtatccgctc	atgagacaat	aaccctgata	aatgcttcaa	taatattgaa	aaaggaagag	2460
tatgagtatt	caacatttcc	gtgtcgccct	tattcccttt	tttgcggcat	tttgccttcc	2520
tgtttttgct	caccagaaaa	cgctgggtgaa	agtaaaaagat	gctgaagatc	agttgggtgc	2580
acgagtgggt	tacatcgaa	tggatctcaa	cagcggtaag	atccttgaga	gttttcgcc	2640
cgaagaacgt	tttccaatga	tgagcacttt	taaagtctct	ctatgtggcg	cggtattatc	2700
ccgtgttgac	gccgggcaag	agcaactcgg	tcgcccgcata	cactattctc	agaatgactt	2760
ggttgagtac	tcaccagtca	cagaaaaagca	tcttacggat	ggcatgacag	taagagaatt	2820
atgcagtgtc	gccataacca	tgagtataaa	cactgcggcc	aacttacttc	tgacaacgat	2880
cggaggaccg	aaggagctaa	ccgctttttt	gcacaacatg	ggggatcatg	taactcgcct	2940
tgatcgttgg	gaaccggagc	tgaatgaagc	cataccaaac	gacgagcgtg	acaccacgat	3000
gcctgtagca	atggcaacaa	actttaact	actattaact	ggcgaactac	ttactctagc	3060
ttccccggca	caatataatag	cgtggatgga	ggcgagataaa	gttgacggac	cacttctgcg	3120
ctcgcccttt	ccggctggct	ggtttattgc	tgataaatct	ggagccgggt	agcgtgggtc	3180
tcgcggtatc	attgcagcac	tggggccaga	tggttaagccc	tcccgtatcg	tagttatcta	3240
cagcagggg	agtcaggcaa	ctatggatga	cagaaataga	cagatcgctg	agatagggtc	3300
ctcactgatt	aagcatttgg	aactgtcaga	ccaagtttac	tcatatatac	tttagattga	3360
tttaaaactt	catttttaat	ttaaaaggat	ctagggtgaag	atcctttttg	ataatctcat	3420
gaccaaatac	ccttaacgtg	agttttcgtt	ccactgagcg	tcagaccccg	tagaaaagat	3480
caaaggatct	tcttgagatc	ctttttttct	gcgcgtaatc	tgctgcttgc	aaacaaaaaa	3540
accaccgcta	ccagcggtgg	tttgtttgcc	ggatcaagag	ctaccaactc	tttttcgaa	3600
ggtaactggc	ttcagcagag	cgcagatacc	aaatactgtc	cttctagtgt	agccgtagt	3660
aggccaccac	ttcaagaact	ctgtagcacc	gcctacatac	ctcgctctgc	taatcctgtt	3720
accagtggct	gctgccagtg	gcgataagtc	gtgtcttacc	gggttgga	caagacgata	3780
gttaccggat	aaggcgagc	ggtcgggctg	aacggggggt	tcgtgcacac	agccagctt	3840
ggagcgaacg	acctacaccg	aactgagata	cctacagcgt	gagctatgag	aaagcgccac	3900
gcttcccga	gggagaaaag	cggacaggtg	tccggttaagc	ggcagggtcg	gaacaggaga	3960
gcgcacgagg	gagcttccag	ggggaaaacgc	ctgggtatctt	tatagtcctg	tcgggtttcg	4020
ccacctctga	cttgagcgtc	gatttttgtg	atgctcgta	ggggggcgga	gcctatggaa	4080
aaacgccagc	aacgcggcct	ttttacggtt	cctggccttt	tgctggcctt	ttgctcacat	4140
gttctttcct	gcgttatccc	ctgattcatt	aatgcaggtc	acgatccttt	ctggcgagtc	4200
cccgtgcgga	gtcggagagc	gctccctgag	cgctgcggc	ccgagaggct	gcgcctggcc	4260
ggccttcggg	ccctcgtgtg	tcccggctgt	aggagggg	ggccgaaaat	gcttccggct	4320
cccgtcttgg	agacacgggc	cggccccctg	cgtgtggcac	ggcgggccgg	gagggcgctc	4380
ccggcccggc	gctgtcccgc	cgtgtgtcct	ggggttgacc	agagggcccc	gggcgctccg	4440
tgtgtggctg	cgatgggtgc	gtttttgggg	acaggtgtcc	gtgtccgtgt	cgcgcgctgc	4500
ctgggcccgc	ggcgtggctg	gtgacgcgac	ctcccggccc	cgggggaggt	atatctttcg	4560
ctccgagtcg	gcattttggg	ccgcggggtt	attagtagaa	acaggggtac		4610

~7048421

<211> 3558

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: PHL2583

<400> 7

tattagtaga	aacaggggtat	tttttattct	agtacattac	gccccgccct	gccactcatc	60
gcagtactgt	tgtaattcat	taagcattct	gccgacatgg	aagccatcac	agacggcatg	120
atgaacctga	atcgccagcg	gcatcagcac	cttgctgcct	tgcgataaat	atttgcccat	180
gggtgaaaacg	ggggcggaaga	agttgtccat	attggccacg	tttaaataca	aactggtgaa	240
actcaccacg	ggattggctg	agacgaaaaa	catattctca	ataaacacct	taggggaaata	300
ggccagggtt	tcaccgtaac	acgccacatc	ttgcgaatat	atgtgtagaa	actgccggaa	360
atcgctcggtg	tattcactcc	agagcgatga	aaacgtttca	gtttgctcat	ggaaaacggg	420
gtaacaagg	tgaacactat	cccatatcac	cagctcaccg	tctttcattg	ccatacggaa	480
ttccggatga	gcattcatca	ggcgggcaag	aatgtgaata	aaggccggat	aaaacttggt	540
cttatttttc	tttacggtct	ttaaaaaggc	cgtaatatcc	agctgaacgg	tctggttata	600
ggtagcggaacg	gcgaggtgact	gaaatgcctc	aaaatgttct	ttacgatgcc	attgggatata	660
atcaacgggtg	gtatatccag	tgattttttt	ctccatgatt	atggccatta	cccttgtttc	720
tactcccccc	caacttcgga	ggtcgaccag	tactccgggc	gaaactttgt	tttttttttt	780
tcccccgatg	ctggaggctg	accagatgtc	cgaaagtgtc	cccccccccc	cccccccccg	840
gcgcggaaacg	gcggggccac	tctggactct	tttttttttt	tttttttttt	tttttgggga	900
tcggccgcta	gcttctgttt	tgccggatga	gagaagattt	tcagcctgat	acagattaaa	960
tcagaacgca	gaagcggctc	gataaaacag	aatttgcctg	gcggcagtag	cgcggtgggtc	1020
ccacctgacc	ccatgccgaa	ctcagaagtg	aaacgccgta	gcgccgatgg	tagtgtgggg	1080
agactgggcc	tttcgtttta	gaactgccag	gcatacaata	aaacgaaaag	ctcagtcgaa	1140
tccgccggga	gcggatttga	tctgtgtgtt	gtcggatgaac	gctctcctga	gtaggacaaa	1200
cccgcgataa	actgccaggc	acgttgcgaa	gcaacggccc	ggagggtggc	gggcaggacg	1260
tgcttttcta	tgattttttt	atcaaattaa	gcagaaggcc	atcctgacgg	atggcctttt	1320
tgagacaata	accctgataa	atgcttcaat	aataattgaa	aagggaagag	atgagtattc	1440
aacattttccg	tgctgcctct	attccctttt	ttgcggcatt	ttgccttctc	gtttttgtct	1500
acccagaaac	gctggtgaaa	gtaaaagatg	ctgaagatca	gttgggtgca	cgagtgggtt	1560
acatcgaaact	ggatctcaac	agcggtaaga	tctttgagag	ttttcgcccc	gaagaacggt	1620
ttccaatgat	gagcactttt	aaagtctctg	tatgtggcgc	ggtattatcc	cgtgttgacg	1680
ccgggcaaga	gcaactcggg	cgccgcatac	actattctca	gaatgacttg	gttgagtact	1740
caccagtcat	agaaaaagcat	cttacggatg	gcattgacagt	aagagaatta	tgcatgtctg	1800
ccataaccat	gagatctcaac	actgcggcca	acttacttct	gacaacgatac	ggaggaccga	1860
aggagctaac	cgcttttttg	cacaacatgg	gggatcatgt	aactcgcctt	gatcgttggg	1920
aaccggagct	gaatgaagcc	ataccaaacg	acgagcgtga	caccacgatg	cctgtagcaa	1980
tggcaacaac	gttgcgcaaa	ctattaactg	gcgaactact	tactctagct	tcccggaac	2040
aattgaataga	ctggatggag	gcggataaaag	tgcaggacc	acttctgcgc	tcggcccttc	2100
cggtctggctg	gtttattgct	gataaatctg	gagccgggtga	gcgtgggtct	cgcggtatca	2160
ttgcagcact	ggggccagat	ggtaaagccct	cccgtatcgt	agttatctac	acgacgggga	2220
gtcaggcaac	tatggatgaa	cgaaatagac	agatcgctga	gataggtgcc	tactgatta	2280
agcattggta	actgtcagac	caagtttact	catatatact	ttagattgat	ttaaaacttc	2340
atttttaatt	taaaaggatc	taggtgaaga	tcctttttga	taatctcatg	accaaatacc	2400
cttaacgtga	gttttcgttc	cactgagcgt	cagaccccgt	agaaaagatc	aaaggatctt	2460
cttgagatcc	ttttttctg	cgcgtaactc	gctgcttgca	aacaaaaaaa	ccaccgtac	2520
cagcgggtggt	ttgttttgctg	gatcaagagc	taccaactct	ttttccgaag	gtaactggct	2580
tcagcagagc	gcagatacca	aatactgtcc	ttctagtgtg	gccgtagtta	ggccaccact	2640
tcaagaactc	tgtagcaccg	cctacatacc	tcgctctgct	aatcctgtta	ccagtggctg	2700
ctgccagtg	cgataagtcg	tgtcttaccg	ggttggactc	aagacgatag	ttaccggata	2760
aggcgagcag	gtcgggctga	acgggggggt	cgtgcacaca	gccagcttg	gagcgaacga	2820
cctacaccga	actgagatac	ctacagcgtg	agctatgaga	aagcgccacg	cttcccgaag	2880
ggagaaaggc	ggacaggtat	ccggtaagcg	gcagggtcgg	aacaggagag	cgcacgagg	2940
agcttccagg	gggaaacg	tggtatcttt	atagtcctgt	cgggtttcgc	cacctctgac	3000
ttgagcgtcg	atttttgtga	tgctcgtcag	ggggcgagg	cctatggaaa	aacgccagca	3060
acgcggcctt	tttacgggtc	ctggcctttt	gctggccttt	tgctcacatg	ttctttctctg	3120
cgttatcccc	tgattcatta	atgcagggtca	cgatcctttc	tggcaggtcc	ccgtgcggag	3180
tcggagagcg	ctccctgagc	gcgtgcggcc	cgagaggtcg	cgcttgcccg	gccttcgggtc	3240
cttcgtgtgt	cccggtcgta	ggagggggccg	gccgaaaatg	cttccggctc	ccgctctgga	3300

~7048421

```

gacacggggc ggccccctgc gtgtggcacg ggccggccgg agggcgctccc cggccccggcg 3360
ctgctcccg cgtgtgtcctg ggggtgacca gagggccccc ggcgctccgt gtgtggctgc 3420
gatggtggcg tttttgggga caggtgtccg tgtccgtgtc gcgcgtcgcc tgggcccggcg 3480
gcgtggctcg tgacgcgacc tcccggcccc gggggaggta tatctttcgc tccgagtcgg 3540
cattttgggc cgccgggt

```

3558

<210> 8

<211> 4343

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: pHL2989

<400> 8

```

ctttctggcg agtccccgtg cggagtcgga gagcgctccc tgagcgctg cggccccgaga 60
ggtcgcgcct ggccggcctt cggtcctcgt tgtgtcccg tcgtaggagg ggccggccga 120
aaatgcttcc ggctcccgt ctggagacac ggccggcccc cctgcgtgtg gcacgggagg 180
ccgggagggg gtccccggcc cggcgctgct cccgcgtgtg tcctgggggt gaccagaggg 240
ccccgggccc tccgtgtgtg gctgcgatgg tggcgtttt ggggacaggt gtccgtgtcc 300
gtgtcgcgcg tcgcctgggc cggcgcgctg gtcggtgacg cgacctccc gccccggggg 360
aggtatatct ttcgctccga attcggcatt tggccgccc gggtattagt agaaacaggg 420
tattttttat actagtaagc tcgaaggagt ccacatgag taaaggagaa gaacttttca 480
ctggagttgt cccaattctt gtgaattag atggtgatgt taatgggcac aaattttctg 540
tcagtggaga gggatgaagg gatgcaacat acggaaaact tacccttaaa tttatttgca 600
ctactggaaa actacctgtt ccatggccaa cactgtcac tactttcact tatggtgttc 660
aatgcttttc aagataccca gatcatatga aacagcatga ctttttcaag agtgccatgc 720
ccgaaggtta tgtacaggaa agaactatat ttttcaaaga tgacgggaac tacaagacac 780
gtgctgaagt caagtttgaa ggtgataccc ttgttaatat aatcgagtta aaaggtattg 840
attttaaaga agatggaaac attcctggag acaaattgga atacaactat aactcacaca 900
atgtatacat catggctgac aagcagaaga acggaatcaa ggccaacttc aagaccgccc 960
acaacatcga ggacggcggc gtgcagctgg ccgaccacta ccagcagaac accccaattg 1020
gcgatggccc tgccttttta ccagacaacc attacctgtc cacacaatct gccctttcga 1080
aagatccaaa cgaaaagaga gaccacatgg tccttcttga gtttgtaaca gctgctggga 1140
ttacacatgg catggatgaa ctatacaagg gatcccatca ccatcaccat cactaagctc 1200
catggtctag atatctagta cattacgccc cgccctgcca ctcatcgag tactgttgta 1260

```

```

attcattaag cattctgccg acatggaagc catcacagac ggcattgatga acctgaatcg 1320
ccagcggcat cagcaccttg tcgccttgcg tataatatat gccatgggtg aaaacggggg 1380
cgaagaagtt gtccatattg gccacgttta aatcaaaact ggtgaaactc acccagggat 1440
tggcactcac aaagaacatg ttctcgatga atcctttagg gaagtaggcc aggttttcac 1500
cgtaaacacg cacaatatgg gaatatatgt gtgaaaactg ccggaatcgc tcgtggtatt 1560
cactccagag cgatgaaaac gtttcagttt gctcatggaa aacgggtgaa caagggtgaa 1620
cactatccca tatcaccagc tcaccgtctt tcattgccat acggaattcc ggatgagcat 1680
tcacagggcg ggcaagaatg tgaataaagg ccgataaaaa cttgtgctta ttttcttta 1740
cggcttttaa aaaggccgta atatccagct gatgccattg ggtataggta cattgagcaa 1800
ctgactgaaa tgcctcaaaa tgttctttac gatgcccatt ggatataatca acggtgggtat 1860
atccagtgat ttttttctcc atgattatgc aaaaaatacc cttgtttcta ctcccccca 1920
acttcggagg tcgaccagta ctccgggcga aactttgttt tttttttttt ccccgatgct 1980
ggaggctcag cagatgtccg aaagtgtccc ccccccccc cccccccggc gcggaacggc 2040
ggggccactc tggactcttt tttttttttt tttttttttt tttggggatc ggccgctagc 2100
ttctgttttg gcggatgaga gaagattttc agcctgatac agattaaatc agaacgcaga 2160
agcggctcta taaaacagaa tttgcctggc ggcagtagcg cgggtgtccc acctgacccc 2220
atgccgaact cagaagtga acgcccgtag gccgatggtg gtgtgggggt tccccatgcg 2280
agatgaggga actgcccagg cagaaaggct agcgaagag cagtcgaaag actgggcctt 2340
tcgttttatc tgtttgttgt cggatgaacgc tctcctgagt aggacaaatc cgccgggagc 2400
ggatttgaa cgttgcaagc aacggcccgg aggggtggcg gcaggacgcc cgccataaac 2460
tgccaggcat caaattaagc agaaggccat cctgacggat ggcctttttg cgtttctaca 2520
aactattttg tttatttttc taaatacatt gaaagagtat gagtattcaa catttccgtg 2580
cctgataaat gcttcaataa tattgaaaaa cgaatgtgta tccgctcatg agacaataac 2640
tcgcccttat tccctttttt gcggcatttt gccttctgt ttttgctcac ccagaaacgc 2700
tggatgaagt aaaagatgct gaagatcagt tgggtgcacg agtgggttac atcgaactgg 2760

```


12

12

12

13

13

Page 13

~7048421

<220>
 <221> variation
 <222> 12...13
 <223> a or g or c or t/u at position 12 or 13

<400> 17
 aguagwaaca rnn 13

<210> 18
 <211> 13
 <212> RNA
 <213> Influenza C virus

<400> 18
 agcaguagca agr 13

<210> 19
 <211> 21
 <212> RNA
 <213> Influenza A virus

<220>
 <221> variation
 <222> 14...16
 <223> a or g or c or t/u at position 14 or 15 or 16

<400> 19
 aguagaaaca aggnnnuuuu u 21

<210> 20
 <211> 21
 <212> RNA
 <213> Artificial Sequence

<220>
 <221> variation
 <222> 14...16
 <223> a or g or c or t/u at position 14 or 15 or 16
 Description of Artificial Sequence: Modified
 influenza A 5'-sequence (pHL1920)

<400> 20
 agaagaauca aggnnnuuuu u 21

<210> 21
 <211> 21
 <212> RNA
 <213> Influenza B virus

<220>
 <221> variation
 <222> 12...16
 <223> a or g or c or t/u at position 12 or 13
 or 14 or 15 or 16

21

19

15

15

<400> 25

nnnnnyguuu cuacu

~7048421

15

<210> 26

<211> 14

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Modified
influenza C 3' sequence

<400> 26

ccccuguuuc uacu

14

WO 00/53786

1

PCT/EP00/01903

SEQUENCE LISTING

<110> ARTEMIS PHARMACEUTICALS. GmbH

5 <120> Stable Recombinant Influenza Viruses Free of Helper
Viruses

<130> 000520wo/JH/ml

10 <140>
<141>

<160> 26

15 <170> PatentIn Ver. 2.1

<210> 1

<211> 4930

20 <212> DNA

<213> Artificial Sequence

<220>

25 <223> Description of Artificial Sequence: pHL2969

<400> 1

	cgtacgaagc	ttctagaggg	attggctgag	acgaaaaaca	tatgctagag	ggattggctg	60
	agacgaaaaa	catatgctag	agcggccgcc	accgcggttg	agctccagct	ttgtttccct	120
	ttagttaggg	ttaattgcgc	gcaggcctag	ctaggtaaag	aaaaatacc	ttgtttctac	180
30	taataaccgc	gcggcccaaa	atgccgactc	ggagcgaaag	atatacctcc	cccggggccg	240
	ggaggtcgcg	tcaccgacca	cgccgcgggc	ccaggcgacg	cgcgacacgg	acacctgtcc	300
	ccaaaaaacg	caccatcgca	gccacacag	gagcgcccg	ggccctctgg	tcaacccag	360
	gacacacgcg	ggagcagcgc	cgggcccggg	acgccctccc	ggccgcccgt	gccacacgca	420
	gggggcccgc	ccgtgtctcc	agagcgggag	ccggaagcat	tttcggccgg	cccctcctac	480
35	gaccgggaca	cacgagggac	cgaaggccgg	ccaggcgcg	cctctcgggc	cgacgcgcgc	540
	ctcagggagc	gctctccgac	tccgcacggg	gactcgccag	aaaggatcgt	gacctgcatt	600
	aatgaatcag	gggataacgc	aggaaagaac	atgtgagcaa	aaggccagca	aaaggccagg	660
	aaccgtaaaa	aggccgcggt	gctggcggtt	ttccataggc	tcggccccc	tgacgagcat	720
	cacaaaaatc	gacgtcaag	tcagaggtgg	cgaaccgga	caggactata	aagataccag	780
40	gcgtttcccc	ctggaagctc	cctcgtgcgc	tctcctgttc	cgaccctgcc	gcttaccgga	840
	tacctgtccg	cctttctccc	ttcggaagc	gtggcgcttt	ctcatagctc	acgctgtagg	900
	tatctcagtt	cggtgtaggt	cgttcgctcc	aagctgggct	gtgtgcacga	accccccggt	960
	cagcccagacc	gctgcgcctt	atccggtaac	tatcgtcttg	agtccaaccc	ggtaagacac	1020
	gacttatcgc	cactggcagc	agccactgg	aacaggatta	gcagagcgag	gtatgtaggc	1080
45	ggtgctacag	agttcttgaa	gtggtggcct	aactacggct	acactagaag	gacagtattt	1140
	ggtatctgcg	ctctgctgaa	gccagttacc	ttcggaaaaa	gagttggtag	ctcttgatcc	1200
	ggcaaacaaa	ccaccgctgg	tagcgggtgg	ttttttgttt	gcaagcagca	gattacgcgc	1260
	agaaaaaaag	gatctcaaga	agatcctttg	atcttttcta	cggggtctga	cgctcagttg	1320
	aacgaaaact	cacgttaagg	gatttttggtc	atgagattat	caaaaaggat	cttcacctag	1380
50	atccttttaa	attaaaaatg	aagtttttaa	tcaatctaaa	gtatatatga	gtaaacttgg	1440
	tctgacagtt	accaatgctt	aatcagtgag	gcacctatct	cagcgatctg	tctatttcgt	1500
	tcatccatag	ttgcctgact	cccgtcgtg	tagataacta	cgatacggga	gggcttacca	1560
	tctggcccca	gtgctgcaat	gataccgcga	gacccacgct	caccggctcc	agatttatca	1620
	gcaataaac	agccagccgg	aaggcccgag	cgcagaagtg	gtcctgcaac	tttatccgcc	1680
55	tccatccagt	ctattaattg	ttgcccggaa	gtagaggtaa	gtagttcgcc	agttatagtg	1740
	ttgcgcaacg	ttgttgccat	tgctacaggc	atcgtgggtg	cacgctcgtc	gtttggtagt	1800
	gcttcattca	gctccggttc	ccaacgatca	aggcgagtta	catgatcccc	catgttgtgc	1860
	aaaaaagcgg	ttagtcctt	cggtccctcc	atcgttgtca	gaagtaagtt	ggccgcagtg	1920
	ttatcactca	tggttatggc	agcactgcat	aattctctta	ctgtcatgcc	atccgtaaga	1980
60	tgcttttctg	tgactggtga	gtactcaacc	aagtcattct	gagaatagtg	tatgcggcga	2040
	ccgagttgct	cttgcccggc	gtcaacacgg	gataataaccg	cgccacatag	cagaacttta	2100

WO 00/53786

2

PCT/EP00/01903

aaagtgtctca tcatttgaaa acgttcttctg gggcgaaaac tctcaaggat cttaccgctg 2160
 ttgagatcca gttcgatgta acccactcgt gcacccaact gatcttcagc atcttttact 2220
 ttcaccagcg tttctgggtg agcaaaaaca ggaaggcaaa atgccgcaaa aaagggaata 2280
 5 agggcgacac ggaaatgttg aatactcata ctcttccttt ttcaatatta ttgaagcatt 2340
 tatcagggtt attgtctcat gagcggatac atatttgaat gtatttagaa aaataaaca 2400
 aagagtttgt agaaacgcaa aaaggccatc cgtcaggatg gccttctgct taatttgatg 2460
 cctggcagtt tatggcgggc gtcttgcccg ccacctccg ggcggttgct tcgcaacgtt 2520
 caaatccgct cccggcggat ttgtctact caggagagcg ttcaccgaca aacaacagat 2580
 10 aaaacgaaag gccagtcctt tcgactgagc ctttcgtttt atttgatgcc tggcagttcc 2640
 ctactctcgc atggggagac cccacactac catcggcgct acggcgtttc acttctgagt 2700
 tcggcatggg gtcagggtggg accaccgcgc tactgccgcc aggcaaattc tgttttatca 2760
 gaccgcttct gcgttctgat ttaatctgta tcaggctgaa aatcttctct catccgcaa 2820
 aacagaagat agcggccgat ccccaaaaaa aaaaaaaaaa gagtccagag 2880
 tggccccgcc gttccgcgcc gggggggggg gggggggggg acactttcgg acatctggtc 2940
 15 gacctccagc atcgggggaa aaaaaaaaaa caaagtttcg cccggagtac tggtcgacct 3000
 ccgaagttgg gggggagtag aaacagggtg gataatcact cactgacgta cgttgagcaa 3060
 ctgactgaaa tgccttgagc aactgactga aatgcctgac gtcttttagca aaagcagggt 3120
 agataatcac tcaactgagtg acatccacat cgtaccagga ttggctgaga cgaaaaacat 3180
 attgtaccag ggattggctg agacgaaaaa catattgtag gtacaaaaat gaacactcaa 3240
 20 atctgtggtt tcgccttgcc ggcagtcac cccacaaatg cagacaaaat ttgtcttgga 3300
 catcatgctg tatcaaatgg caccaaagta aacacactca ctgagagagg agtagaagt 3360
 gtcaatgcaa cggaacagtg ggagcggaca aacatcccca aaatttgctc aaaagggaaa 3420
 agaaccactg atcttgacca atgcggactg ttagggacca ttaccggacc acctcaatgc 3480
 25 gaccaatttc tagaattttc agctgatcta ataatcgaga gacgagaagg aaatgatgtt 3540
 tgttaccggt ggaagtttgt taatgaagag gcattgcgac aaatcctcag aggatcaggt 3600
 gggattgaca aagaacaat gggattcaca tatagtggaa taaggaccaa cggaacaact 3660
 agtgcattga gaagatcagg gtcttcattc tatgcagaaa tggagtggct cctgtcaaat 3720
 acagacaatg cttctttccc acaaatgaca aaatcataca aaaacacagg gagagaatca 3780
 gctctgatat tctggggaat ccaccattca ggatcaacca ccgaacagac caaactatat 3840
 30 gggagtgga ataaactgat aacagtcggg agttccaaat atcatcaatc ttttgtgccg 3900
 agtccaggaa cacgaccgca gataaatggc cggtccggac ggattgattt tcattgggtg 3960
 atcttgatc ccaatgatac agttactttt agtttcaatg gggctttcat agctccaaat 4020
 cgtgccagct tcttgagggg aaagtccatg gggatccaga gcgatgtgca ggttgatgct 4080
 aattgcgaag gggaatgcta ccacagtggg gggactataa caagcagatt gccttttcaa 4140
 35 aacataaata gcagagcagt tggcaaatgc ccaagatatg taaaacagga aagtttatta 4200
 ttggcaactg ggatgaagaa ccttccaaaa aaaggaaaaa aaggaggcctg 4260
 tttggtgcta tagcagggtt tattgaaaat ggttgggaag gtctgggtcg cgggtggtac 4320
 ggtttcaggc atcagaatgc acaaggagaa ggaactgcag cagactacaa aagcacccaa 4380
 40 tcggcaattg atcagataac cggaaggtta aatagactca ttaagaaaac caaccagcaa 4440
 tttgagctaa tagataatga attcactgaa gtggaaaagc agattggcaa tttaattaac 4500
 tggaccaaag actccatcac agaagtatgg tcttacaatg ctgaacttct tgtggcaatg 4560
 gaaaaccagc aactatttga tttggctgat tcagagatga acaagctgta tgagcgagt 4620
 aggaacaatg taaggaaaaa tgctgaagag gatggcactg gttgctttga aatttttcat 4680
 45 aaatgtgacg atgattgtat ggctagtata aggaacaata cttatgatca cagcaaatc 4740
 agagaagaag cgatgcaaaa tagaatacaa attgaccag tcaaattgag tagtggctac 4800
 aaagatgtga tactttggtt tagcttcggg gcacatgct ttttgcttct tgccattgca 4860
 atgggccttg ttttcatatg tgtgaagaac ggaaacatgc ggtgcactat atgcatttaa 4920
 agcttgcattg 4930

<210> 2

<211> 5811

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: pAM403

<400> 2

60 aattcctttg cctaatttaa atgaggactt aacctgtgga aatattttga tgtgggaagc 60
 tgttactgtt aaaactgagg ttattggggt aactgctatg ttaaacttgc attcagggac 120

WO 00/53786

3

PCT/EP00/01903

	acaaaaaact	catgaaaatg	gtgctggaaa	accatttcaa	gggtcaaatt	ttcatttttt	180
	tgctgttggt	ggggaacctt	tggagctgca	gggtgtgtta	gcaaactaca	ggaccaata	240
	tctgtctcaa	actgtaaccc	caaaaaatgc	tacagttgac	agtcagcaga	tgaacactga	300
5	ccacaaggct	gttttgata	aggataatgc	ttatccagtg	gagtgctggg	ttcctgatcc	360
	aagtaaaaat	gaaaacacta	gatatttttg	aacctacaca	gggtgggaaa	atgtgcctcc	420
	tgttttgac	attactaaca	cagcaaccac	agtgtctctt	gatgagcagg	gtgttgggcc	480
	cttggtcaaa	gctgacagct	tgtatgtttc	tgctgttgac	atttgtgggc	tgtttaccac	540
	cacttctgga	acacagcagt	ggaagggaact	tcccagatat	tttaaaatta	cccttagaaa	600
	gcggtctgtg	aaaaacccct	acccaatttc	ctttttgtta	agtgcactaa	ttaacaggag	660
10	gacacagagg	gtggatgggc	agcctatgat	tggatgtcc	tctcaagtag	aggaggtag	720
	ggtttatgag	gacacagagg	agcttcctgg	ggatccagac	atgataagat	acattgatga	780
	gtttggacaa	accacaacta	gaatgcagt	aaaaaaatgc	tttattttgtg	aaattttgtg	840
	tgtattgtct	ttatttgaat	ccattataag	gtgaataaaa	caagttaaca	acaacaattg	900
	cattcattttt	atgtttcagg	ttcaggggga	gggtgtgggag	gttttttaaa	gcaagtaaaa	960
15	cctctacaaa	tgtggtatgg	ctgattatga	tctctagtca	aggcactata	catcaaatat	1020
	tccttattaa	cccctttaca	aattaaaaag	ctaaaggtag	acaatttttg	agcatagtta	1080
	ttaatagcag	acactctatg	cctgtgtgga	gtaagaaaaa	acagtatgtt	atgattataa	1140
	ctgttatgcc	tacttataaa	ggttacagaa	tattttttcca	taattttctt	gtatagcagt	1200
	gcagcttttt	cctttgtggt	gtaaatagca	aagcaagcaa	gagttctatt	actaaacaca	1260
20	gcagtactca	aaaaacttag	caattctgaa	ggaaggtcct	tggggtcttc	tacctttctc	1320
	ttcttttttg	gaggagtaga	atgttgagag	tcagcagtag	cctcatcatc	actagatggc	1380
	atcttctctg	agcaaaacag	gttttctctca	ttaaaggcat	tccaccactg	ctcccattca	1440
	tcagttccat	aggttggaat	ctaaaataca	caaacaatta	gaatcagtag	tttaacacat	1500
	tatacactta	aaaattttat	atctacctta	gagctttaaa	tctctgtagg	tagtttgtcc	1560
25	aattatgtca	caccacagaa	gtaagggtcc	ttcacaaaga	tccgggacca	aagcggccat	1620
	cgtgcctccc	cactcctgca	gttcgggggc	atggtgcgc	ggatagccgc	tgctggtttc	1680
	ctggatgccg	acggatttgc	actgcgggta	gaactccgcg	aggtcgtcca	gcctcaggca	1740
	gcagctgaac	caactcgcga	ggggatcgag	cccgggggtg	gcgaagaact	ccagcatgag	1800
	atccccgcgc	tggaggatca	tccagccggc	gtcccggaaa	acgattccga	agcccaacct	1860
30	ttcatagaag	gcggcggtgg	aatcgaaatc	tctgtatggc	aggttgggag	tcgcttgggc	1920
	ggtcatttctg	atgaattcga	gctcggtacc	cggggatcct	ctagaggcat	ttcagtttctg	1980
	tcctcacgga	ctcatcagag	ttgctcaatt	cgaacccag	agtcccgtc	agaagaactc	2040
	gtcaagaagg	cgatagaagg	cgatgcgctg	cgaatcgga	gcggcgatag	cgtaagcac	2100
	gaggaagcgg	tcagccatt	cgccgccaag	ctcttcagca	atatcacggg	tagccaacgc	2160
35	tatgtcctga	tagcgggtccg	ccacacccag	ccggccacag	tcgatgaatc	cagaaaagcg	2220
	gccattttcc	accatgatat	tcggcaagca	ggcatcgcca	tgggtcacga	cgagatcctc	2280
	gccgtcgggc	atgcgcgcct	tgagcctggc	gaacagttcg	gctggcgcg	gcccctgatg	2340
	ctcttcgtcc	agatcatcct	gatcgacaag	accggcttcc	atccgagtac	gtgctcgctc	2400
	gatgcgatgt	ttcgcttggt	ggtcgaaatg	gcaggtagcc	ggatcaagcg	tatgcagccg	2460
40	ccgcatttga	tcagccatga	tggtaacttt	ctcggcagga	gcaaggtag	atgacaggag	2520
	atcctgcccc	ggcacttcgc	ccaatagcag	ccagtcctt	cccgtttcag	tgacaacgtc	2580
	gagcacagct	gcgcaaggaa	cgccgctcgt	ggccagccac	gatagccgcg	ctgcctcgctc	2640
	ctgcagttca	ttcagggcac	cggacaggtc	ggtcttgaca	aaaagaaccg	ggcgcccctg	2700
	cgctgacagc	cggaacacgg	cggcatcaga	gcagccgatt	gtctgtttgtg	cccagtcata	2760
45	gccgaatagc	ctctccaccc	aagcggccgg	agaacctgcg	tgcaatccat	cttgttcaat	2820
	catgcgaaac	gatcctcatc	ctgtctcttg	atcagatctt	gatcccctgc	gccatcagat	2880
	ccttggcggc	aagaaaagcca	tccagtttac	tttgacgggc	ttcccaacct	taccagaggg	2940
	cgccccagct	ggcaattccg	gttcgcttgc	tgtccataaa	accgcccagt	ctagctatcg	3000
	ccatgtaagc	ccactgcaag	ctacctgctt	tctcttttgcg	cttgcgtttt	cccttgctca	3060
50	gatagcccag	tagctgacat	tcatccgggg	tcagcaccgt	ttctgcggac	tggctttcta	3120
	cgtgttccgc	ttccttttagc	agcccttgcg	ccctgagtgc	ttgcggcagc	gtgaagcttt	3180
	tgtgcaaaagc	ctaggcctcc	aaaaaagcct	cctcactact	tctggaatag	ctcagaggcc	3240
	gaggcggcct	cgccctctgc	ataaataaaa	aaatattagt	agccatggg	cggagaatgg	3300
	gcggaactgg	gcggagttag	ggcggggatg	ggcggagtta	ggggcgggac	tatggttgc	3360
55	gactaattga	gatgcatgct	ttgcatactt	ctgcctgctg	gggagcctgg	ggactttcca	3420
	cacctggttg	ctgactaatt	gagatgcatg	ctttgcatac	ttctgcctgc	tggggagcct	3480
	ggggactttc	cacaccctaa	ctgacacaca	ttccacagct	gcctcgcgcg	tttcggtgat	3540
	gacggtgaaa	acctctgaca	catgcagctc	ccggagacgg	tcacagcttg	tctgtaagcg	3600
	gatgccggga	gcagacaagc	ccgtcagggc	gcgtcagcgg	gtgttggcgg	gtgtcgggc	3660
60	gcagccatga	cccagtcacg	tagcgatagc	ggagtgtata	ctggcttaac	tatgcggcat	3720
	cagagcagat	tgtactgaga	gtgcaccata	tgcgggtgtga	aataccgcac	agatgcgtaa	3780

WO 00/53786

4

PCT/EP00/01903

5 ggagaaaata ccgcatcagg cgctcttccg ctctcctcgt cactgactcg ctgcgctcgg 3840
 tcgttcgggt gcggcgagcg gtatcagctc actcaaaggc ggtaatacgg ttatccacag 3900
 aatcagggga taacgcagga aagaacatgt gagcaaaagg ccagcaaaag gccaggaacc 3960
 gtaaaaaggc cgcgttgctg gcgtttttcc ataggctccg ccccttgac gagcatcaca 4020
 5 aaaaatcgacg ctcaagtcag aggtggcgaa actataaaga taccaggcgt 4080
 ttccccctgg aagctccctc gtgcgctctc ctgttccgac cctgccgctt accggatacc 4140
 tgtccgcctt tctcccttcg ggaagcgtgg cgcttttctc tagctcacgc tgtaggtatc 4200
 tcagttcgggt gtaggtcgtt cgtcccaagc tgggctgtgt gcacgaaccc cccgttcagc 4260
 ccgaccgctg cgccttatcc ggtaactatc gtcttgagtc caaccggta agacacgact 4320
 10 tatcgccact ggcagcagcc actggtaaca ggattagcag agcgaggtat gtaggcggtg 4380
 ctacagagtt cttgaagtgg tggcctaact acggctacac tagaaggaca gtatttggtg 4440
 tctgcgctct cgtgaagcca gttaccttcg gaaaaagagt tggtagctct tgatccggca 4500
 aacaaaccac cgctggtagc ggtgggtttt ttgtttgcaa gcagcagatt acgcgagaa 4560
 15 aaaaaggatc tcaagaagat cctttgatct tttctacggg gtctgacgct cagtggaaacg 4620
 aaaactcacg ttaagggtat ttggtcatga gattatcaaa aaggatcttc acctagatcc 4680
 ttttaaatta aaaatgaagt tttaaatcaa tctaaagtat atatgagtaa acttggtctg 4740
 acagttacca atgcttaatc agtgaggcac ctatctcagc gatctgtcta tttcgttcat 4800
 ccatagttgc ctgactcccc gtcgtgtaga taactacgat acgggagggc ttaccatctg 4860
 20 gccccagtgc tgcaatgata ccgcgagacc cagctcacc ggctccagat ttatcagcaa 4920
 taaaccagcc agccggaagg gccgagcgca gaagtggtec tgcaacttta tccgcctcca 4980
 tccagtctat taattgttgc cggaagcta gagtaagtag ttcgccagtt aatagtttgc 5040
 gcaacgttgt tgccattgct gcaggcatcg tgggtgtcag ctctcgtgtt ggtatggctt 5100
 cattcagctc cggttcccaa cgatcaaggc gagttacatg atcccccatg ttgtgcaaaa 5160
 aagcggttag ctcttcgggt cctccgatcg ttgtcagaag taagttggcc gcagtgttat 5220
 25 cactcatggt tatggcagca ctgcataatt ctcttactgt catgccatcc gtaagatgct 5280
 tttctgtgac tgggtgagtac tcaaccaagt cattctgaga atagtgtatg cggcgaccga 5340
 gttgctcttg cccggcgcta acacgggata ataccgcgc acatagcaga actttaaaag 5400
 tgctcatcat tggaaaacgt tcttcggggc gaaaactctc aaggatctta ccgctctta 5460
 gatccagttc gatgtaaccc actcgtgcac ccaactgatc ttcagcatct tttactttca 5520
 30 ccagcgtttc tgggtgagca aaaacaggaa ggcaaaatgc cgcaaaaaag ggaataaggg 5580
 cgacacggaa atggtgaata ctcatactct tcttttttca atattattga agcatttatc 5640
 aggggttattg tctcatgagc ggatacatat ttgaatgtat ttagaaaaat aaacaaatag 5700
 gggttccgcg cacatttccc cgaaaagtgc cacctgacgt ctaagaaacc attattatca 5760
 35 tgacattaac ctataaaaaat aggcgtatca cgaggccctt tcgtcttcaa g 5811

<210> 3

<211> 2005

<212> DNA

40 <213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: vHM41

45 <400> 3

agtagaaaca aggggtatttt tctttaccta gctaggcctg cgcgcaatta accctcacta 60
 aagggaaaca aagctggagc tccaccgcgg tggcgccgc tctagcatat gtttttcgtc 120
 tcagccaatc cctctagcat atgtttttcg tctcagccaa tccctctaga agcttcgtac 180
 50 gcatgcaagc tttaaatgca tatagtgcac cgcatgtttc cgttcttcac acatatgaaa 240
 acaaggccca ttgcaatggc aagaagcaaa aagcatgatg ccccgaaagt aaaccaaaag 300
 atcacatctt tgtagccact actcaatttg actgggtcaa tttgtattct attttgcatc 360
 gcttcttctc tgtatttgct gtgatcataa gtattgttcc ttatactagc catacaatca 420
 tcgtcacatt tatgaaaaat ttcaaagcaa ccagtgccat cctcttcagc attttccctt 480
 aattgttttc tcaactcgctc atacagcttg ttcattctctg aatcagccaa atcaatagtg 540
 55 tgctgggttt ccattgccac aagaagtcca gcattgtaag accatacttc tgtgatggag 600
 tctttggctc agttaattaa attgccaate tgcttttcca cttcagttaa ttcattatct 660
 attagctcaa attgctgggt ggttttctta atgagctat ttaactttcc ggttatctga 720
 tcaattgccg attgggtgct tttgtagtct ctgcagttc cttctccttg tgcattctga 780
 tgctgaaac cgtaccaccc gtcgaccaga ccttcccaac cattttcaat aaaccctgct 840
 60 atagaccaa acaggcctct ttttttccct tttttggaag gttcgggaac gttcttcac 900
 ccagttgcca ataataaact ttctgtttt acatatcttg ggcatttgcc aactgctctg 960

WO 00/53786

5

PCT/EP00/01903

```

ctatttatgt tttgaaaagg caatctgctt gttatagtcc ctccactgtg gtagcattcc 1020
ccttcgcaat tagcatcaac ctgcacatcg ctctggatcc ccatggactt tcccctcaag 1080
aagctggcac gatttggagc tatgaaagcc ccattgaaac taaaagtaac tgtatcattg 1140
ggatccaaga tcaaccaatg aaaatcaatc cgtccggacc ggccatttat ctgcggtcgt 1200
5 gttccctggac tcggcacaaa agattgatga tatttggAAC tcccgaactgt tatcagttta 1260
tttccactcc catatagttt ggtctgttcg gtggttgatc ctgaatggtg gattccccag 1320
actatcagag ctgattctct cctctgtgtt ttgtatgatt ttgtcatttg tgggaaagaa 1380
gcattgtctg tatttgacag gagccactcc atttctgcat agaatgaaga cctgatctt 1440
ctacatgcac tagttgttcc gttggtcctt attccactat atgtgaatcc cattgtttct 1500
10 ttgtcaatcc cactgatcc tctgaggatt tgtcgcaatg cctcttcatt aacaaacttc 1560
cccgggtaac aaacatcatt tccttctcgt ctctcgatta ttagatcagc tgaaaattct 1620
agaaattggc cgcattgagg tggtcggta atgggcccta acagtcgcga ttggccaaga 1680
tcagtggttc ttttcccttt tgagcaaatt ttggggatgt ttgtccgctc cactgtttcc 1740
gttgacattga caacttctac tcctctctca gtgagtgtgt ttacttttgt gccatttgat 1800
15 acagcatgat gtccaagaca aattttgtct gcatttgttg ggatgactgc cgcaagggcg 1860
aaaaccagga tttgagtgtt catttttggt cctacaatat gtttttcgtc tcagccaatc 1920
cctggtacaa tatgtttttc gtctcagcca atcctggtac gatgtggatg tcaactcagt 1980
agtgattatc taccctgctt ttgct 2005

```

```

<210> 4
<211> 1146
<212> DNA
<213> Artificial Sequence

```

```

<220>
<223> Description of Artificial Sequence: vHM81

```

```

<400> 4
30 agtagaaaca agggatattt tctttaccta gctaggcctg cgcgcaatta accctcacta 60
aagggaacaa aagctggagc tccaccgcgg tggcggccgc tctagcatat gtttttcgtc 120
tcagccaatc cctctagcat atgtttttcg tctcagccaa tccctctaga agcttcgtac 180
gcattgcttaa ataagctgaa acgagaaagt tcttatctct tgctccactt caagcggtag 240
ttgtaaggct tgcataaatg ttatttggtc aaaactattc tctgttatct tcaatctatg 300
35 tctcacttct tcaattaacc atcttatttc ttcaaatttc tgactcaatt gttctcgcca 360
ttttccgttt ctgctttgga gggagtggag gtccccatt ctccattactg ctctctccaa 420
cgaatctctg tatagtttca gagactcgaa ctgtgttatc attccattca agtcctccga 480
tgaggacccc aattgcattt ttgacatcct catcagtatg tcttggaaga gaaggcaatg 540
gtgaaatttc gccgacaatt gctccctcat cgggttaaagc ccttaatagt atgagagttt 600
40 ccagccgatc gaaaatcaca ctgaagtttg ctttcagtat gatgttcttc cccatgatcg 660
cctggtccat tctgatgcaa agggagcctg ccactttctg tttgggcatg agcatgaacc 720
agtcccttga catctcttca agagtcatgt cagttaggta gcgtgtagca ggtacagagg 780
caatggtcac tttaagtgcc tcacgggatt cgtcctccag aatccgctcc actatctgct 840
ttccaacacg agtagctgtg tcgatgtcca gaccaagagt gctgcctctt cccctcaggg 900
45 acttctgatc tcggcgaaagt cgggtcaagga atggggcatc acccatttct tgggtctgcaa 960
atcgtttgcg gacatgccaa agaaagcagt ctacctgaaa gcttgacaca gtgttggaat 1020
ccattatggg acctacaata tgtttttcgt ctcagccaat ccttggtaca atatgttttt 1080
cgtctcagcc aatcctggtg cgatgtggat gtcactcagt gagtgttat ctaccctgct 1140
50 tttgct 1146

```

```

<210> 5
<211> 5860
<212> DNA
55 <213> Artificial Sequence

```

```

<220>
<223> Description of Artificial Sequence: pAM424

```

```

60 <400> 5
catcgattgg ctgactgatg agtccgtgag gacgaaacga aaaacatatt gtagagctcg 60

```

WO 00/53786

6

PCT/EP00/01903

	aattcatcga	aatgaccgac	caagcgacgc	ccaacctgcc	atcacgagat	ttcgattcca	120
	ccgcccgcctt	ctatgaaagg	ttgggcttcg	gaatcgtttt	ccgggacgcc	ggctggatga	180
	tcctccagcg	cggggatctc	atgctggagt	tcttcgccc	ccccgggctc	gatccccctcg	240
5	cgagttggtt	cagctgctgc	ctgaggctgg	acgacctcgc	ggagttctac	cggcagtgca	300
	aatccgtcgg	catccaggaa	accagcagcg	gctatccgcg	catccatgcc	cccgaactgc	360
	aggagtgggg	aggcacgatg	gccgctttgg	tcccggaatc	ttgtgaagga	accttacttc	420
	tgtggtgtga	cataattgga	caaaactacct	acagagattt	aaagctctaa	ggtaaataata	480
	aaatttttaa	gtgtataatg	tgtaaacta	ctgattctaa	ttgtttgtgt	attttagatt	540
10	ccaacctatg	gaactgatga	atgggagcag	tggtggaatg	cctttaatga	ggaaaacctg	600
	ttttgctcag	aagaaatgcc	atctagtgat	gatgaggcta	ctgctgactc	tcaacattct	660
	actcctccaa	aaaagaagag	aaaggtagaa	gaccccaagg	actttccttc	agaattgcta	720
	agttttttga	gtcatgctgt	gtttagtaat	agaactcttg	cttgctttgc	tatttacacc	780
	acaaaggaaa	aagctgcact	gctatacaag	aaaattatgg	aaaaatattc	tgtaaccttt	840
	ataagtaggc	cataacagta	taatacatac	atactgtttt	ttcttactcc	acacaggcat	900
15	agagtgtctg	ctattaataa	ctatgctcaa	aaattgtgta	cctttagctt	tttaatttgt	960
	aaaggggtta	ataaggaata	tttgatgtat	agtgccttga	ctagagatca	taatcacgca	1020
	taccacattt	gtagaggttt	tacttgcttt	aaaaaacctc	ccacacctcc	ccctgaacct	1080
	gaaacataaa	atgaatgcaa	ttgttggtgt	taacttgttt	attgcagctt	ataatggtta	1140
20	caaataaagc	aatagcatca	caaatttcac	aaataaagca	tttttttcac	tgcatcttag	1200
	ttgtggtttg	tccaaactca	tcaatgtatc	ttatcatgtc	tggtatcccca	ggaagctcct	1260
	ctgtgtcctc	ataaacctca	acctcctcta	cttgagagga	cattccaatc	ataggctgcc	1320
	catccacctc	ctgtgtcctc	ctgttaatta	ggtcacttaa	caaaaaggaa	attgggtagg	1380
	ggtttttcac	agaccgcttt	ctaagggtaa	ttttaaaata	tctgggaagt	cccttcact	1440
25	gctgtgttcc	agaagtgttg	gtaaacagcc	cacaaatgtc	aacagcagaa	acatacaagc	1500
	tgtcagcttt	gcacaagggc	ccaacacctc	gctcatcaag	aagcactgtg	gttgctgtgt	1560
	tagtaatgtg	caaaacagga	ggcacatttt	ccccacctgt	gtaggttcca	aaatatctag	1620
	tgttttcatt	tttacttgga	tcaggaaccc	agcactccac	tggtataagca	ttatccttat	1680
	ccaaaacagc	cttggtgtca	gtgttcactc	gctgactgtc	aactgtagca	ttttttgggg	1740
30	ttacagtttg	agcaggatat	ttggctcctg	agtttgctaa	cacaccctgc	agctccaaag	1800
	gttccccacc	aacagcaaaa	aaatgccttg	ttgacccttg	aatgggtttt	ccagcaccat	1860
	tttcatgagt	tttttgtgtc	cctgaatgca	agtttaacat	agcagttacc	ccaataacct	1920
	cagttttaac	agtaacagct	tcccacatca	aaatatattc	acagggttaag	tcctcattta	1980
	aattaggcaa	aggaattctt	gaagacgaaa	gggcctcgtg	atacgcttat	ttttataggt	2040
35	taatgtcatg	ataataatgg	tttcttagac	gtcaggtggc	acttttcggg	gaaatgtgcg	2100
	cggaaccctc	atltgtttat	ttttctaaat	acattcaaat	atgtatccgc	tcatgagaca	2160
	ataaccctga	taaaatgctc	aataatatgt	aaaaagggaag	agtatgagta	ttcaacattt	2220
	ccgtgtcgcc	cttattccct	tttttcggcg	atlttgctt	cctgtttttg	ctcaccacga	2280
	aacgctgggtg	aaagtaaaaag	atgctgaaga	tcagttgggt	gcacgagtgg	gttacatcga	2340
40	actggatctc	aacagcggtg	agatccttga	gagttttcgc	cccgaagaac	gttttccaat	2400
	gatgagcact	tttaaagttc	tgctatgtgg	cgcggtatta	tcccgtgttg	acgccgggca	2460
	agagcaactc	ggtcgccgca	tacactattc	tcagaatgac	ttggttgagt	actcaccagt	2520
	cacagaaaag	catcttacgg	atggcatgac	agtaagagaa	ttatgcagtg	ctgccataac	2580
	catgagtgat	aacactgcgg	ccaacttact	tctgacaacg	atcggaggac	cgaaggagct	2640
	aaccgctttt	ttgcacaaca	tgggggatca	tgtaactcgc	cttgatcgtt	gggaaccgga	2700
45	gctgaatgaa	gccataccaa	acgacgagcg	tgacaccacg	atgcctgcag	caatggcaac	2760
	aacgttgctc	aaactattaa	ctggcgaaact	acttactcta	gcttcccggc	aacaattaat	2820
	agactggatg	gaggcggata	aagttgcagg	accacttctg	cgctcggccc	ttccggctgg	2880
	ctggtttatt	gctgataaat	ctggagccgg	tgagcgtggg	tctcgcggta	tcattgcagc	2940
	actggggcca	gatggtaagc	cctcccgtat	cgtagtattc	tacacgacgg	ggagtcaggc	3000
50	aactatggat	gaacgaaata	gacagatcgc	tgagataggt	gcctcactga	tttaagcattg	3060
	gtaactgtca	gaccaagttt	actcatatat	acttttagatt	gattttaaacc	ttcattttta	3120
	attttaaagg	atctaggtga	agatcctttt	tgataatctc	atgacaaaaa	tcccttaacg	3180
	tgagttttcg	ttccactgag	cgtcagaccc	cgtagaaaag	atcaaaagat	cttcttgaga	3240
	tccttttttt	ctgcgcgtaa	tctgctgctt	gcaaacaata	aaaccaccgc	taccagcggt	3300
55	ggtttgtttg	ccggatcaag	agctaccaac	tctttttccg	aaggtaactg	gcttcagcag	3360
	agcgcagata	ccaaataactg	tccttctagt	gtagccgtag	ttaggccacc	acttcaagaa	3420
	ctctgtagca	ccgcctacat	acctcgctct	gctaactcctg	ttaccagtgg	ctgctgccag	3480
	tggcgataag	tcgtgtctta	ccgggttggg	ctcaagacga	tagttaccgg	ataaggcgca	3540
	gcggtcgggg	tgaacggggg	gttcgtgcac	acagcccagc	ttggagcgaa	cgacctacac	3600
60	cgaactgaga	tacctacagc	gtgagctatg	agaaagcgcc	acgcttcccg	aaggagaaaa	3660
	ggcggacagg	tatccggtaa	gcggcagggg	cggaaacagga	gagcgcacga	gggagcttcc	3720

WO 00/53786

7

PCT/EP00/01903

5 agggggaaac gcctggtatc tttatagtc tgtcgggttt cgccacctct gacttgagcg 3780
 tcgattttttg tgatgctcgt caggggggag gagcctatgg aaaaacgcca gcaacgcggc 3840
 ctttttaacgg ttccctggcct tttgctggcc ttttgctcac atgttctttc ctgcgttatc 3900
 ccctgattct gtggataacc gtattaccgc ctttgagtga gctgataccg ctgcgcgcag 3960
 ccgaacgacc gagcgcagcg agtcagtgag cgaggaagcg gaagagcgcc tgatgcggta 4020
 ttttctcctt acgcatctgt gcggtatttc acaccgcata tgggtgcactc tcagtacaat 4080
 ctgctctgat gccgcatagt taagccagta tacactccgc tatcgctacg tgactgggtc 4140
 atggctgcgc cccgacaccc gccaacaccc gctgacgcgc cctgacgggc ttgtctgctc 4200
 ccggcatccg cttacagaca agctgtgacc gtctccggga gctgcatgtg tcagaggttt 4260
 10 tcaccgtcat caccgaaacg cgcgaggcag ctgtggaatg tgtgtcagtt aggggtgtgga 4320
 aagtcccccag gtcctccagc aggcagaagt atgcaaagca tgcattctca ttagtcagca 4380
 accaggtgtg gaaagtcccc aggtcccca gcaggcagaa gtatgcaaag catgcatctc 4440
 aattagtcag caaccatagt cccgccccta actccgcca tcccggccct aactccgcc 4500
 agttccgccc atttccgccc ccatggctga ctaatttttt ttattttatgc agaggccgag 4560
 15 gccgcctcgg cctctgagct attccagaag tagtgaggag gcttttttgg aggcctaggc 4620
 ttttgcaaaa agcttcacgc tgccgcaagc actcagggcg caagggctgc taaaggaagc 4680
 ggaacacgta gaaagccagt ccgcagaaac ggtgctgacc ccggatgaat gtcagctact 4740
 gggctatctg gacaaggga aacgcaagcg caaagagaaa gcaggtagct tgcagtgggc 4800
 20 ttacatggcg atagctagac tggcggttt tatggacagc aagcgaaccg gaattgccag 4860
 ctggggcgcc ctctggtaag gttgggaagc cctgc aaagt aaactggatg gctttcttgc 4920
 cgccaaggat ctgatggcgc aggggatcaa gatctgatca agagacagga tgaggatcgt 4980
 ttgcgatgat tgaacaagat ggattgcacg caggttctcc ggccgcttgg gtggagaggc 5040
 tattcggcta tgactgggca caacagacaa tcggctgctc tgatgccgcc gtgttccggc 5100
 tgtcagcgca gggcgcccg gttctttttg tcaagaccga cctgtccggt gccctgaatg 5160
 25 aactgcagga cgaggcagcg cggctatcgt ggctggccac gacgggcgtt ccttgccgag 5220
 ctgtgctcga cgttgtcact gaagcgggaa gggactggct gctattgggc gaagtgccgg 5280
 ggcagatct cctgtcatct caccttgctc ctgcagaaa agtatccatc atggctgatg 5340
 caatgcggcg gctgcatacg cttgatccgg ctacctgcc attcgaccac caagcgaaac 5400
 atcgcatcga gcgagcacgt actcggatgg aagccggtct tgtcgatcag gatgatctgg 5460
 30 acgaagagca tcaggggctc gcgccagccg aactgttcgc caggctcaag gcgcgcacgc 5520
 ccgacggcga ggatctcgtc gtgacccatg gcgatgcctg cttgccgaat atcatggtgg 5580
 aaaatggccg cttttctgga ttcacgcact gtggccggct ggggtgtggc gaccgctatc 5640
 aggacatagc gttggctacc cgtgatattg ctgaagagct tggcgcgcaa tgggctgacc 5700
 gcttcctcgt gctttacggt atcgccgctc ccgattcgca gcgcacgcgc ttctatcgcc 5760
 35 ttcttgacga gttcttctga gcgggactct ggggttcgaa tcctaccagg gattggctga 5820
 ctgatgagtc cgtgaggacg aaacgaaaaa catatggtac 5860

<210> 6

40 <211> 4610

<212> DNA

<213> Artificial Sequence

<220>

45 <223> Description of Artificial Sequence: pHL2507

<400> 6

50 gaggcatctt agtcagttgc tcaaggtacc aaaatgaaca ctcaaactct ggttttccgc 60
 cttgcggcag tcatcccccac aaatgcagac aaaatttgtc ttggacatca ttgtgtatca 120
 aatggcacca aagtaaacac actcactgag agaggagtag aagttgtcaa tgcaacggaa 180
 acagtggagc ggacaaacat ccccaaaatt tgctcaaaaag ggaaaagaac cactgatctt 240
 ggccaatgcg gactgttagg gaccattacc ggaccacctc aatgcgacca atttctagaa 300
 ttttcagctg atctaataat cgagagacga gaaggaaatg atgtttgtta cccggggaag 360
 55 tttgttaatg aagaggcatt gcgacaaatc ctgagaggat cagggtggat tgacaaagaa 420
 acaatgggat tcacatatag tggaataagg accaacggaa caactagtgc atgtagaaga 480
 tcagggtctt cattctatgc agaaatggag tggctcctgt caaatacaga caatgcttct 540
 ttcccacaaa tgacaaaatc atacaaaac acaggagag aatcagctct gatagcttg 600
 ggaatccacc attcaggatc aaccaccgaa cagaccaaac tatatgggag tggaataaaa 660
 ctgataacag tcgggagttc caaatatcat caatcttttg tgccgagtc aggaacacga 720
 60 ccgcagataa atggccggtc cggacggatt gattttcatt ggttgatctt ggatcccaat 780
 gatacagtta cttttagttt caatggggct ttcatagctc caaatcgtgc cagcttcttg 840

WO 00/53786

8

PCT/EP00/01903

	aggggaaagt	ccatggggat	ccagagcgat	gtgcagggtt	atgctaattg	cgaaggggaa	900
	tgctaccaca	gtggagggac	tataacaagc	agattgcctt	ttcaaaacat	aaatagcaga	960
	gcagttggca	aatgcccacg	atatgtaaaa	caggaaagtt	tattattggc	aactgggatg	1020
	aagaacgttc	ccgaaccttc	caaaaaaagg	aaaaaaagag	gcctgttttg	tgctatagca	1080
5	gggttttattg	aaaatgggtg	ggaagggtctg	gtcgacgggt	ggtacgggtt	caggcatcag	1140
	aatgcacaag	gagaaggaac	tgcagcgagc	tacaaaagca	cccaatcggc	aattgatcag	1200
	ataaccggaa	agttaaatag	actcattaag	aaaaccaacc	agcaatttga	gctaatatag	1260
	aatgaattca	ctgaagtggg	aaagcagatt	ggcaatttaa	ttaactggac	caaagactcc	1320
	atcacagaag	tatggtctta	caatgctgaa	cttcttggtg	caatggaaaa	ccagcacact	1380
10	attgattttg	ctgattcaga	gatgaacaag	ctgtatgagc	gagtggaggaa	acaattaagg	1440
	gaaaatgctg	aagaggatgg	cactggttgc	tttgaaaattt	ttcataaatg	tgacgatgat	1500
	tgatatggcta	gtataaggaa	caatacttat	gatcacagca	aatacacaga	agaagcgatg	1560
	caaaatagaa	tacaaattga	cccagtcaaa	ttgagttagt	gctacaaaaga	tgtgatactt	1620
	tggttttagct	tcggggcctc	atgctttttg	cttcttgcca	ttgcaatggg	ccttggtttc	1680
15	atatgtgtga	agaacggaaa	catgcggtgc	actatttgta	tataggtttg	gaaaaaaaca	1740
	ccccctgttt	ctactccccc	ccaacttcgg	aggctcgacca	gtactccggg	cgaacttttg	1800
	tttttttttt	ttcccccgat	gctggaggtc	gaccagatgt	ccgaaagtgt	ccccccccc	1860
	ccccccccc	ggcgcggaac	ggcggggcca	ctctggactc	tttttttttt	tttttttttt	1920
	ttttttgggg	atcgcccgct	agcttctggt	ttggcggatg	agagaagatt	ttcagcctga	1980
20	tacagattaa	atcagaacgc	agcagcggtc	tgataaaaca	gaatttgccct	ggcggcagta	2040
	gegcggtggt	cccacctgac	cccatgcccga	actcagaagt	gaaacgcccgt	acgcgcagtg	2100
	gtagtgtggg	gtctccccc	gcgagagtag	ggaactgcca	ggcatcaaat	aaaacgaaag	2160
	gctcagtcga	aagactgggc	ctttcggttt	atctgttggt	tgctcggtgaa	cgctctcctg	2220
	agtaggacaa	atccgcccgg	agcggatttg	aacgttgcca	agcaacggcc	cggagggtgg	2280
25	cgggcaggac	gccccccata	aactgccagg	catcaaatta	agcagaaggc	catcctgacg	2340
	gatggccttt	ttgcgtttct	acaaaactctt	ttgtttattt	ttctaaatac	attcaaatat	2400
	gtatccgctc	atagacaat	aaccctgata	aatgcttcaa	taatattgaa	aaaggaagag	2460
	tatgagtatt	caacatttcc	gtgtcgccct	tattcccttt	tttgcggcat	tttgccctcc	2520
	tgtttttgct	cacccagaaa	cgctgggtgaa	agtaaaagat	gctgaagatc	agttgggtgc	2580
30	acgagtgggt	tacatcgaac	tggatctcaa	cagcggtaag	atccttgaga	gttttcgccc	2640
	cgaagaacgt	tttccaatga	tgagcacttt	taaagttctg	ctatgtggcg	cggtattatc	2700
	ccgtgttgac	gcccgggcaag	agcaactcgg	tcgccgcata	cactattctc	agaatgactt	2760
	ggttgagtac	tcaccagtcg	cagaaaagca	tcttacggat	ggcatgacag	taagagaatt	2820
	atgcagtgct	gccataacca	tgagtgataa	cactgcggcc	aacttacttc	tgacaacgat	2880
35	cggaggaccg	aaggagctaa	ccgctttttt	gcacaaac	ggggatcatg	taactcgctt	2940
	tgatcggttg	gaaccggagc	tgaatgaagc	cataccaaac	gacgagcggt	acaccagatg	3000
	gcctgtagca	atggcaacaa	cgttgcgcaa	actattaact	ggcgaactac	ttactctagc	3060
	ttcccggcaa	caattaatag	actggatgga	ggcgataaaa	gttgacaggac	cacttctgctg	3120
	ctcgccctt	ccggctggct	ggtttattgc	tgataaatct	ggagccgggtg	agcgtgggtc	3180
40	tcgcggtatc	attgcagcac	tggggccaga	tggtaaagccc	tcccgtatcg	tagttatcta	3240
	cacgacgggg	agtcaggcaa	ctatggatga	acgaaataga	cagatcgctg	agatagggtc	3300
	ctcactgatt	aagcattggt	aactgtcaga	ccaagtttac	tcatatatac	tttagattga	3360
	tttaaaactt	catttttaat	ttaaaaggat	ctagtgaaag	atcctttttg	ataatctcat	3420
	gacccaaatc	ccttaacgtg	agttttcggt	ccactgagcg	tcagaccccg	tagaaaaag	3480
45	caaaggatct	tcttgagatc	ctttttttct	gcgcgtaatc	tgctgcttgc	aaacaaaaaa	3540
	accaccgcta	ccagcgggtg	tttgtttgcc	ggatcaagag	ctaccaactc	tttttccgaa	3600
	ggttaactggc	ttcagcagag	cgcagatacc	aaatactgtc	cttctagtgt	agccgtagtt	3660
	aggccaccac	ttcaagaact	ctgtagcacc	gcctacatac	ctcgctctgc	taatcctgtt	3720
	accagtggct	gctgccagtg	gcgataagtc	gtgtcttacc	gggttggaact	caagacgata	3780
50	gttacccggat	aaggcgagc	ggtcgggctg	aacggggggt	tcgtgcacac	agcccagctt	3840
	ggagcgaacg	acctacaccg	aactgagata	ctacagcggt	gagctatgag	aaacgcccac	3900
	gcttcccga	gggagaaagg	cggacaggta	tccggttaagc	ggcaggggtc	gaacacgaga	3960
	gcgcacgagg	gagcttccag	ggggaacgc	ctggatctct	tatagtcctg	tcgggtttcg	4020
	ccacctctga	cttgagcgtc	gattttttgtg	atgctcgtca	ggggggcgga	gcctatggaa	4080
55	aaacgccagc	aacgcggcct	ttttacggtt	cctggccttt	tgctggcctt	ttgctcacat	4140
	gttctttcct	gcgttatccc	ctgattcatt	aatgcaggtc	acgatecctt	ctggcgagtc	4200
	cccgtgcgga	gtcggagagc	gtccctgag	cgcgtgcggc	ccgagaggtc	gcgcctggcc	4260
	ggccttcggt	ccctcgtgtg	tcccggctgt	aggaggggcc	ggccgaaaat	gcttccggct	4320
	cccgtctctg	agacacgggc	cggccccctg	cgtgtggcac	gggcggccgg	gagggcgtcc	4380
60	ccggcccggc	gctgctccc	cgtgtgtcct	ggggttgacc	agagggcccc	gggcgctccg	4440
	tgtgtggctg	cgatggtggc	gtttttgggg	acaggtgtcc	gtgtccgtgt	cgcgcgctcg	4500

ctgggcccgc ggcgtggtcg gtgacgcgac ctcccggccc cgggggaggt atatctttcg 4560
ctccgagtcg gcattttggg ccgcccgggtt attagtagaa acaggggtac 4610

- 5 <210> 7
<211> 3558
<212> DNA
<213> Artificial Sequence

- 10 <220>
<223> Description of Artificial Sequence: pHL2583

<400> 7

15	tattagtaga	aacagggtat	tttttattct	agtacattac	gccccgccct	gccactcatc	60
	gcagtactgt	tgtaattcat	taagcattct	gccgacatgg	aagccatcac	agacggcatg	120
	atgaacctga	atcgccagcg	gcatacagcac	cttgctgcct	tgcgtataat	atttgcccat	180
	ggtgaaaacg	ggggcggaaga	agttgtccat	attggccacg	tttaaataca	aactggtgaa	240
	actcaccacg	ggattggctg	agacgaaaaa	catattctca	ataaaccctt	tagggaaata	300
	ggccagggtt	tcaccgtaac	acgccacatc	ttgcgaatat	atgtgtagaa	actgccggaa	360
20	atcgctgtgg	tattcactcc	agagcgatga	aaacgtttca	gtttgctcat	ggaaaacggg	420
	gtaacaaggg	tgaacactat	cccatatcac	cagctcaccg	tctttcattg	ccatacggaa	480
	ttccggatga	gcattcatca	ggcgggcaag	aatgtgaata	aaggccggat	aaaacttggtg	540
	cttatttttc	tttacgggtc	ttaaaaaggc	cgtaatatcc	agctgaacgg	tctggttata	600
	ggtacattga	gcaactgact	gaaatgcctc	aaaatgttct	ttacgatgcc	attgggatat	660
25	atcaacgggtg	gtatatccag	tgattttttt	ctccatgatt	atggccatta	cccttggttc	720
	tactcccccc	caacttcgga	ggtcgaccag	tactccgggc	gaaactttgt	tttttttttt	780
	tcccccgatg	ctggagggtcg	accagatgtc	cgaaagtgtc	cccccccccc	cccccccccg	840
	gcgcggaacg	gcggggccac	tctggactct	tttttttttt	tttttttttt	tttttgggga	900
	tcggccgcta	gcttctgttt	tggcggatga	gagaagattt	tcagcctgat	acagattaaa	960
30	tcagaacgca	gaagcgggtc	gataaaaacag	aatttgctcg	gcggcagtag	cgcggtggtc	1020
	ccacctgacc	ccatgccgaa	ctcagaagtg	aaacgccgta	gcgccgatgg	tagtgtgggg	1080
	tctccccatg	cgagagtagg	gaactgccag	gcatacaata	aaacgaaagg	ctcagtcgaa	1140
	agactggggc	tttcgtttta	tctgtttgtt	gtcgggtgaac	gctctcctga	gtaggacaaa	1200
	tccgcgggga	gcggatttga	acgttgcgaa	gcaacggccc	ggagggtggc	gggcaggacg	1260
35	cccgcataaa	actgccaggc	atcaaatata	gcagaaggcc	atcctgacgg	atggcctttt	1320
	tgcgtttcta	caaactcttt	tgtttatttt	tctaaatata	ttcaaataatg	tatccgctca	1380
	tgagacaata	accctgataa	atgcttcaat	aatattgaaa	aaggaagagt	atgagtattc	1440
	aacattttccg	tgctgccect	attccctttt	ttgcggcatt	ttgccttctc	gtttttgtct	1500
40	acccagaaac	gctggtgaaa	gtaaaagatg	ctgaagatca	gttggttgca	cgagtgggtt	1560
	acatcgaaact	ggatctcaac	agcggtaaga	tccttgagag	ttttcgcccc	gaagaacggt	1620
	ttccaatgat	gagcactttt	aaagttctgc	tatgtggcgc	ggtattatcc	cgtgttgacg	1680
	ccgggcaaga	gcaactcggg	cgccgcatac	actattctca	gaatgacttg	gttgagtact	1740
	caccagtcac	agaaaagcat	cttagcgatg	gcatagcagt	aagagaatta	tgacgtgctg	1800
	ccataaccat	gagtgataac	actgcggcca	acttacttct	gacaacgatc	ggagggaccg	1860
45	aggagctaac	cgcttttttg	cacaacatgg	gggatcatgt	aactcgcctt	gatcgttggg	1920
	aaccggagct	gaatgaagcc	ataccaaacg	acgagcgtga	caccacgatg	cctgtagcaa	1980
	tggcaacaac	gttgcgcaaa	ctattaactg	gcgaactact	tactctagct	tcccggcaac	2040
	aattaataga	ctggatggag	gcggataaag	ttgcaggacc	acttctgcgc	tcggcccttc	2100
	cggctggctg	gtttatttgt	gataaatctg	gagccgggtga	gcgtgggtct	cgcggtatca	2160
50	ttgcagcat	ggggccagat	ggtaagccct	cccgtatcgt	agttatctac	acgacgggga	2220
	gtcaggcaac	tatggatgaa	cgaaatagac	agatcgctga	gataggtgac	tcactgatta	2280
	agcattggta	actgtcagac	caagtttact	catatatact	ttagattgat	ttaaaacttc	2340
	atttttaatt	taaaaggatc	taggtgaaga	tcctttttga	taatctcatg	acaaaaatcc	2400
	cttaacgtga	gttttcgttc	cactgagcgt	cagaccccg	agaaaagatc	aaaggatctt	2460
55	cttgagatcc	tttttttctg	cgcgtaatct	gctgcttgca	aacaaaaaaa	ccaccgctac	2520
	cagcggtggt	ttgtttgccc	gatcaagagc	taccaactct	ttttccgaag	gtaactggct	2580
	tcagcagagc	gcagatacca	aatactgtcc	ttctagtgtg	gccgtagtta	ggccaccact	2640
	tcaagaactc	tgtagcaccg	cctacatacc	tcgctctgct	aatcctgtta	ccagtggtg	2700
	ctgccagtg	cgataagtcg	tgtcttaccg	ggttggactc	aagacgatag	ttaccggata	2760
60	aggcgcagcg	gtcgggctga	acgggggggt	cgtgcacaca	gcccagcttg	gagcgaacga	2820
	cctacaccga	actgagatac	ctacagcgtg	agctatgaga	aagcgcacac	cttcccgaag	2880

WO 00/53786

10

PCT/EP00/01903

	ggagaaaggc	ggacaggtat	cgggtaagcg	gcagggctcg	aacaggagag	cgcacgaggg	2940
	agcttccagg	gggaaacgcc	tggtatcttt	atagtctctgt	cgggtttcgc	cacctctgac	3000
	ttgagcgtcg	atTTTTgtga	tgctcgtcag	ggggcgagg	cctatggaaa	aacgccagca	3060
	acgcggcctt	tttacggttc	ctggcctttt	gctggccttt	tgctcacatg	ttctttcctg	3120
5	cgttatcccc	tgattcatta	atgcaggtca	cgatcctttc	tggcgagtc	ccgtgcggag	3180
	tcggagagcg	ctccctgagc	gcgtgcggcc	cgagaggtcg	cgcttgcccg	gccttcggtc	3240
	cctcgtgtgt	cccggtcgta	ggaggggccc	gccgaaaatg	cttcgggtc	ccgctctgga	3300
	gacacgggcc	ggccccctgc	gtgtggcacg	ggcgccggg	agggcgcccc	cggcccggcg	3360
	ctgctccgcg	gtgtgtcctg	gggttgacca	gagggccccg	ggcgctccgt	gtgtggctgc	3420
10	gatggtggcg	tttttgggga	caggtgtccg	tgtccgtgtc	gcgcgtcgcc	tgggccggcg	3480
	gcgtggctcg	tgacgcgacc	tcccggcccc	gggggaggt	tatctttcgc	tccgagtcgg	3540
	cattttgggc	cgccgggt					3558

15 <210> 8
 <211> 4343
 <212> DNA
 <213> Artificial Sequence

20 <220>
 <223> Description of Artificial Sequence: pHL2989

	<400> 8						
	ctttctggcg	agtccccgtg	cggagtcgga	gagcgctccc	tgagcgcgtg	cggccccgaga	60
25	ggtcgcgcct	ggccggcctt	cggctccctcg	tgtgtcccg	tcgtaggagg	ggccggccga	120
	aaatgcttcc	ggctcccgt	ctggagacac	gggcccggcc	cctgcgtgtg	gcacgggccc	180
	ccgggagggc	gtccccggcc	cggcgctgct	cccgcgtgtg	tcctgggggt	gaccagaggg	240
	ccccgggcgc	tccgtgtgtg	gctgcgatgg	tgcgctttt	ggggacagg	gtccgtgtcc	300
	gtgtcgcgcg	tcgcctgggc	cggcgcgctg	gtcggtgacg	cgacctccc	gccccggggg	360
30	aggtatatct	ttcgctccga	gtcggcattt	tgggcccgcg	ggttattagt	agaaacaggg	420
	tattttttat	actagtaagc	tcgaaggagt	ccaccatgag	taaaggagaa	gaacttttca	480
	ctggagttgt	cccaattctt	gttgaattag	atggtgatgt	taatgggcac	aaattttctg	540
	tcagtggaga	gggtgaagg	gatgcaacat	acggaaaact	tacccttaaa	tttatttgca	600
	ctactggaaa	actacctgtt	ccatggccaa	cacttgtcac	tactttcact	tatggtgttc	660
35	aatgcttttc	aagataccca	gatcatatga	aacagcatga	ctttttcaag	agtgccatgc	720
	ccgaaggtta	tgtacaggaa	agaactatat	ttttcaaa	tgacgggaac	tacaagacac	780
	gtgctgaagt	caagtgtgaa	gggtgataccc	ttgttaatag	aatcgagtta	aaaggatttg	840
	attttaaaga	agatggaaac	attcttggtg	acaaattgga	atacaactat	aactcacaca	900
	atgtatacat	catggctgac	aagcagaaga	acggaatcaa	ggccaacttc	aagaccgccc	960
40	acaacatcga	ggacggcggc	gtgcagctgg	ccgaccacta	ccagcagaac	accccaattg	1020
	gcgatggccc	tgtcctttta	ccagacaacc	attacctgtc	cacacaatct	gccctttcga	1080
	aagatcccaa	cgaaaagaga	gaccacatgg	tccttcttga	gtttgaaca	gctgtggga	1140
	ttacacatgg	catggatgaa	ctatacaaag	gatccccatca	ccatcaccat	cactaagctc	1200
	catggtctag	atatctagta	cattacgccc	cgccttgcca	ctcatcgag	tactgttgta	1260
45	attcattaag	cattctgccg	acatggaagc	catcacagac	ggcatgatga	acctgaatcg	1320
	ccagcggcat	cagcaccttg	tcgccttgcg	tataatattt	gcccatgggtg	aaaacggggg	1380
	cgaagaagtt	gtccatattg	gccacgttta	aatcaaaact	ggtgaaactc	acccagggat	1440
	tggcactcac	aaagaacatg	ttctcgatga	atccttttag	gaagtaggcc	aggttttcac	1500
	cgtaacacgc	cacatcttgc	gaatatatgt	gtagaaactg	ccggaatcg	tcgtggtatt	1560
50	cactccagag	cgatgaaaac	gtttcagttt	gctcatggaa	aacggtgtaa	caagggtgaa	1620
	cactatccca	tatcaccagc	tcaccgtctt	tcattgccat	acggaattcc	ggatgagcat	1680
	tcatacggcg	ggcaagaatg	tgaataaagg	ccggataaaa	cttgtgctta	ttttctttta	1740
	cggctcttta	aaaggccgta	atatccagct	gaacggtctg	gttataggta	cattgagcaa	1800
	ctgactgaaa	tgcttcaaaa	tgttctttac	gatgccattg	ggatatatca	acggtgggtat	1860
55	atccagtgat	ttttttctcc	atgattatgc	aaaaaatacc	cttgttttcta	ctcccccca	1920
	acttcggagg	tcgaccagta	ctccgggcga	aactttgttt	tttttttttc	ccccgatgct	1980
	ggaggctcgac	cagatgtccg	aaagtgtccc	cccccccccc	cccccccggc	gcggaacggc	2040
	ggggccactc	tggactcttt	tttttttttt	tttttttttt	tttggggatc	ggccgctagc	2100
	ttctgttttg	gcggatgaga	gaagattttc	agcctgatac	agattaaatc	agaacgcaga	2160
60	agcggctctga	taaaacagaa	tttgccctggc	ggcagtagcg	cgggtgggtccc	acctgacccc	2220
	atgccgaact	cagaagtga	acgccgtagc	gccgatggta	gtgtgggggtc	tccccatgcg	2280

WO 00/53786

PCT/EP00/01903

11

	agagtaggga	actgccaggc	atcaaataaa	acgaaaggct	cagtcgaaag	actgggcctt	2340
	tcgttttata	tggtgtttgt	cggtgaacgc	tctcctgagt	aggacaaatc	cgccggggagc	2400
	ggatttgaac	gttgcggaagc	aacggcccgc	aggggtggcg	gcaggacgcc	cgccataaac	2460
	tgccaggcat	caaattaagc	agaaggccat	cctgacggat	ggcctttttg	cgtttctaca	2520
5	aactcctttg	tttatttttc	taaatacatt	caaatatgta	tccgctcatg	agacaataac	2580
	cctgataaat	gcttcaataa	tattgaaaaa	ggaagagtat	gagtattcaa	catttccgtg	2640
	tcgcccttat	tccctttttt	gcggcatttt	gccttccctg	ttttgctcac	ccagaaacgc	2700
	tggtgaaagt	aaaagatgct	gaagatcagt	tggtgacacg	agtgggttac	atcgaactgg	2760
	atctcaacag	cggttaagatc	cttgagagtt	ttcgccccga	agaacgtttt	ccaatgatga	2820
10	gcacttttaa	agttctgcta	tgtggcgcg	tattatcccg	tggtgacgcc	gggcaagagc	2880
	aactcggtcg	ccgcatacac	tattctcaga	atgacttgg	tgagtactca	ccagtcacag	2940
	aaaagcatct	tacggatggc	atgacagtaa	gagaattatg	cagtgtgcc	ataaccatga	3000
	gtgataaac	tgccggccaac	ttacttctga	caacgatcgg	aggaccgaag	gagctaaccg	3060
	cttttttgca	caacatgggg	gatcatgtaa	ctcgccttga	tcgttgggaa	ccggagctga	3120
15	atgaagccat	accaaacgac	gagcgtgaca	ccacgatgcc	tgtagcaatg	gcaacaactg	3180
	tgcgcaaaact	attaactggc	gaactactta	ctctagcttc	ccggcaacaa	ttaatagact	3240
	ggatggaggc	ggataaaagt	gcaggaccac	ttctgcgtc	ggcccttccg	gctggctggt	3300
	ttattgctga	taaatctgga	gccggtgagc	gtgggtctcg	cggtatcatt	gcagcactgg	3360
	ggccagatgg	taagccctcc	cgtatcgtag	ttatctacac	gacggggagt	caggcaacta	3420
20	tggtgaaacg	aatagacag	atcgctgaga	taggtgcctc	actgattaag	cattggtaac	3480
	tgtcagacca	agtttactca	tatatacttt	agattgattt	aaaacttcat	ttttaattta	3540
	aaaggatcta	ggtgaagatc	cttttttgata	atctcatgac	caaaatccct	taacgtgagt	3600
	tttcggtcca	ctgagcgtca	gaccccgtag	aaaagatcaa	aggatcttct	tgagatcctt	3660
	tttttctgcy	cgtaatctgc	tgcttgcaaa	caaaaaaacc	accgctacca	gcggtggttt	3720
25	gtttgccgga	tcaagagcta	ccaactcttt	ttccgaaggt	aactggcttc	agcagagcgc	3780
	agataccaaa	tactgtcctt	ctagtgtagc	cgtagttagg	ccaccacttc	aagaactctg	3840
	tagcaccgcc	tacatacctc	gctctgctaa	tcctgttacc	agtggctgct	gccagtggcg	3900
	ataagtcgtg	tcttaccggg	ttggactcaa	gacgatagtt	accggataag	gcgcagcgg	3960
	cggtgctgaac	gggggggttcg	tgcacacagc	ccagcttggg	gcgaacgacc	tacaccgaac	4020
30	tgagataacct	acagcgtgag	ctatgagaaa	gcgccacgct	tcccgaagg	agaaaggcgg	4080
	acaggatatcc	ggtaagcggc	agggtcggaa	caggagagcg	cacgaggagg	cttcaggggg	4140
	gaaacgcctg	gtatctttat	agtcctgtcg	ggtttcgcca	cctctgactt	gagcgtcgat	4200
	ttttgtgatg	ctcgtcagg	ggcgaggacc	tatggaaaaa	cgccagcaac	gcggcctttt	4260
	tacggttcct	ggccttttgc	tggccttttg	ctcacatggt	ctttcctgcy	ttatccctg	4320
35	attcattaat	gcaggtcacg	atc				4343

<210> 9

<211> 3888

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: pHL1920

<400> 9

	cccaaaaaaa	aaaaaaaaaa	aaaaaaaaag	agtccagagt	ggccccgcgc	ttccgcgcgc	60
	gggggggggg	ggggggggga	cacttttcgga	catctgggtc	acctccagca	tcgggggaaa	120
	aaaaaaaaaac	aaagtttcgc	ccggagtact	ggtcgacctc	cgaagttggg	ggggagtaga	180
50	aacagggttag	ataatcactc	actgagtgc	atccacatcg	cgagcgcgcg	taatacgact	240
	cactataggg	cgaattgggt	accgggcccc	ccctcgaggt	cgacggtatc	gataagcttc	300
	gacgagattt	tcaggagcta	aggaagctaa	aatggagaaa	aaaatcactg	gatataccac	360
	cgttgatata	tcccattggc	atcgtaaaga	acattttgag	gcatttcagt	cagttgtctc	420
	atgtacctat	aaccagaccg	ttcagctgga	tattacggcc	tttttaaaga	ccgtaaagaa	480
55	aaataagcac	aagttttatc	cggcctttat	tcacattctt	gcccgcctga	tgaatgctca	540
	tccggaattc	cgtatggcaa	tgaaagacgg	tgagctgggt	atatgggata	gtgttcaccc	600
	ttgttacacc	gttttccatg	agcaaaactga	aacgttttca	tcgctctgga	gtgaatacca	660
	cgacgatttc	cggcagtttc	tacacatata	ttcgcaagat	gtggcgtggt	acggtgaaaa	720
	cctggcctat	ttccctaaag	ggtttattga	gaatatgttt	ttcgtctcag	ccaatccctg	780
60	ggtgagtttc	accagttttg	atttaaacgt	ggccaatatg	gacaacttct	tcgcccccg	840
	tttcaccatg	ggcaaatatt	atcgcgaagg	cgacaaggtg	ctgatgccgc	tgccgattca	900

5 gggttcatcat gccgtttgtg atgggttcca tgtcggcaga atgcttaatg aattacaaca 960
 gtactgcat gagtggcagg gcggggcgta attttttttaa ggcagttatt ggtgccctta 1020
 aacgcctggt gctacgcctg aataagtgat aataagcgga tgaatggcag aaattcgtcg 1080
 aagcttgata tcgaattcct gcagcccggg ggatccacta gttctagagc ggccgccacc 1140
 gcggtggagc tccagctttt gtcccttta gtgagggtta attgcgcgca ggcctagcta 1200
 ggtaaagaaa aatacccttg attcttctaa taaccggcg gcccataatg ccgactcgga 1260
 gcgaaagata tacctcccc ggggccggga ggtcgcgtca ccgaccacgc cgccggccca 1320
 ggagacgcgc gacacggaca cctgtcccca acacgcggga catcgagcc acacaggag 1380
 cgcccggggc cctctggtca accccaggac acacgcggga gcagcgccg gccgggacg 1440
 10 cctcccggc cgcccggtgc acacgcaggg ggccggcccg tgtctccaga gcgggagccg 1500
 gaagcatttt cgcccgggcc ctcctacgac cgggacacac gagggaccga aggccggcca 1560
 ggcgcgacct ctggggcgcc acgcgcgctc agggagcgct ctccgactcc gcacggggac 1620
 tcgccagaaa ggatcgtgac ctgcattaat gaatcagggg ataacgcagg aaagaacatg 1680
 tgagcaaaa gcccagcaaaa ggccaggaac cgtaaaaagg ccgcgttgct ggcgtttttc 1740
 15 cataggctcc gcccctctga cgagcatcac aaaaatcgac gctcaagtca gaggtggcga 1800
 aaccgcagag gactataaag ataccaggcg ttccccctg gaagctccct cgtgcgtct 1860
 cctgttccga cctgcgcgt tactcggtac ctgtccgct tctcccttc gggagcggtg 1920
 gcgctttctc atagctcacg ctgtaggat ctcagttcgg tgtaggtcgt tcgctccaag 1980
 ctgggctgtg tgcacgaacc ccccgttcag cccgaccgct gcgccttatc cggtaactat 2040
 20 cgtcttgagt ccaaccgggt aagacacgac ttatcgccac tggcagcagc cactggtaac 2100
 aggattagca gagcgaggta tgtaggcggt gctacagagt tcttgaagtg gtggcctaac 2160
 tacggctaca ctagaaggac agtatttggt atctgcgtc tgcgaagcc agttacctc 2220
 ggaaaaagag ttggtagctc tactcgcgaga aaaaaaggat ctcaagaaga tcctttgatc 2280
 25 tttgtttgca agcagcagat tactcgcgaga gaaaactcac gttaagggat tttgggtcatg 2400
 tttctacgg ggtctgacgc tcagtggaa cttttaaatt aaaaatgaag ttttaaataca 2460
 agattatcaa aaaggatctt cacctagatc gacagttacc aatgcttaat cagtgaggca 2520
 atctaaagta tatatgagta aacttggtct tccatagttg cctgactccc cgtcgtgtag 2580
 cctatctcag cgatctgtct atttcgttca ggccccagtg ctgcaatgat accgcgagac 2640
 30 ataactaca tacgggaggg cttaccatct ataccagca ataccagtg ttaattggtg ccgggaagct 2700
 ccacgctcac cggctccaga ttatcagca atccagtcta ttaattggtg ccgggaagct 2760
 agaagtggtc ctgcaacttt atccgcctcc cgcaacggtg ttgccattgc tacaggcatc 2820
 agagtaagta gttcgccagt taatagtttg cgttcagct ccggttccca acgatcaagg 2880
 gtggtgtcac gtcgctggt ttggtatggc tcattcagct gctccttcg tcctccgac 2940
 35 cgagttacat gatcccccat gttgtgcaaa aaagcggtta tcaactcatg ttatggcagc actgcataat 3000
 gttgtcagaa gtaagttggc cgagtggtta ttttctgtga ctggtgagta ctcaaccaag 3060
 tctcttactg tcatgccatc cggtcgaccg agttgctct gcccggcgtc aacacgggat 3120
 tcattctgag aatagtgtat aactttaaaa gtgctcatca ttggaaaacg ttcttcgggg 3180
 aataccgcgc cacatagcag accgctggtg agatccagtt cgatgtaacc cactcgtgca 3240
 40 cgaaaactct caaggatctt ttttactttc accagcggtt ctgggtgagc aaaaacagga 3300
 cccaactgat cttcagcatc ttttactttc accagcggtt ctgggtgagc aaaaacagga 3360
 aggcaaatg ccgcaaaaaa gggaataagg gcgacacgga aatgttgaat actcatactc 3420
 ttcctttttc aatattattg aagcatttat caggggtatt gtctcatgag cggatacata 3480
 tttgaatgta tttagaaaaa taaacaaaag agtttgtaga aacgcaaaaa ggccatccgt 3540
 45 caggatggcc ttctgcttaa tttgatgcct ggcagtttat ggcgggcgtc ctgcccgcga 3600
 cctccgggc cgttgcttcg caacgttcaa atccgctccc ggcggatttg tctactcag 3660
 gagagcgttc accgacaaac aacagataaa acgaaaggcc cagtctttcg actgagcctt 3720
 tcgttttatt tgatgcctgg cagttcccta ctctcgcatg gggagacccc acactaccat 3780
 cggcgctacg gcgtttcact tctgagttcg gcatggggtc aggtgggacc accgcgctac 3840
 50 tgccgccagg caaattctgt tttatcagac cgcttctgcg ttctgattta atctgtatca 3888
 ggctgaaaat cttctctcat ccgcaaaaac agaagctagc ggccgatc

<210> 10

<211> 12

55 <212> RNA

<213> Influenza A virus

<400> 10

ccugcuuuug cu

60

12

WO 00/53786

PCT/EP00/01903

13

5 <210> 11
 <211> 12
 <212> RNA
 <213> Influenza B virus
 <400> 11
 nnygcuucug cu 12

10 <210> 12
 <211> 12
 <212> RNA
 <213> Influenza C virus

15 <400> 12
 ccugcuucug cu 12

20 <210> 13
 <211> 12
 <212> RNA
 <213> Artificial Sequence

25 <220>
 <223> Description of Artificial Sequence: Modified
 influenza A 3' sequence (pHL1104 and 1920)
 <400> 13
 ccuguuucua cu 12

30 <210> 14
 <211> 12
 <212> RNA
 <213> Artificial Sequence

35 <220>
 <223> Description of Artificial Sequence: Modified
 influenza A 3' sequence (pHL1948)
 <400> 14
 ccucguucuc cu 12

45 <210> 15
 <211> 13
 <212> RNA
 <213> Artificial Sequence

50 <220>
 <223> Description of Artificial Sequence: Modified
 influenza A 5' sequence (pHL1920)
 <400> 15
 agaagaaucagg 13

60 <210> 16
 <211> 13
 <212> RNA
 <213> Influenza A virus

WO 00/53786

14

PCT/EP00/01903

<400> 16
aguagaaaca agg 13

5
<210> 17
<211> 13
<212> RNA
<213> Influenza B virus

10
<400> 17
aguagwaaca rnn 13

15
<210> 18
<211> 13
<212> RNA
<213> Influenza C virus

20
<400> 18
agcaguagca agr 13

25
<210> 19
<211> 21
<212> RNA
<213> Influenza A virus

30
<400> 19
aguagaaaca aggnnnuuuu u 21

35
<210> 20
<211> 21
<212> RNA
<213> Artificial Sequence

40
<220>
<223> Description of Artificial Sequence: Modified
influenza A 5'-sequence (pHL1920)

45
<400> 20
agaagaauca aggnnnuuuu u 21

50
<210> 21
<211> 21
<212> RNA
<213> Influenza B virus

55
<400> 21
aguagwaaca rnnnnuuuu u 21

60
<210> 22
<211> 19
<212> RNA
<213> Artificial Sequence

60
<220>
<223> Description of Artificial Sequence: Modified

WO 00/53786

15

PCT/EP00/01903

influenza C 5' sequence

5 <400> 22
aguaguaaca agrguuuuu 19

10 <210> 23
<211> 15
<212> RNA
<213> Artificial Sequence

15 <220>
<223> Description of Artificial Sequence: Modified
influenza A 3' sequence (pHL1104 and 1920)

<400> 23
nnnccucuuu cuacu 15

20 <210> 24
<211> 15
<212> RNA
<213> Artificial Sequence

25 <220>
<223> Description of Artificial Sequence: Modified
influenza A 3' sequence (pHL1948)

30 <400> 24
nnnccucguu cuccu 15

35 <210> 25
<211> 15
<212> RNA
<213> Artificial Sequence

40 <220>
<223> Description of Artificial Sequence: Modified
influenza B 3' sequence

<400> 25
nnnnnyguuu cuacu 15

45 <210> 26
<211> 14
<212> RNA
<213> Artificial Sequence

50 <220>
<223> Description of Artificial Sequence: Modified
influenza C 3' sequence

55 <400> 26
ccccuguuuc uacu 14

21/pvb

Stable Recombinant Influenza Viruses Free of Helper Viruses**5 Field of the Invention**

The invention relates to a recombinant influenza virus for high-yield expression of incorporated foreign gene(s), which is genetically stable in the absence of any helper virus; a method for the production of said recombinant influenza virus, a method for constructing specific ribozyme-sensitive influenza carrier strains; pharmaceutical compositions comprising
10 said recombinant influenza viruses; and the use of said recombinant influenza virus for preparing medicaments for vaccination purposes.

Technical Background

15 Redesigning influenza virus into a vector system for expression of foreign genes similar to what has been achieved in several other thoroughly studied viruses such as adenovirus, retrovirus, Semliki Forest virus or Rabies virus has the advantage of an industrially well established mode of cheap propagation for influenza in fertilized chicken eggs leading to rather
20 high titers (above 10^{10} /ml). On the other hand none of its genes may be deleted from the influenza genome according to our present knowledge, and give room for large-size foreign insertions. Only small fragments of foreign polypeptide chains such as B cell epitopes (10 to 15 amino acids) may be inserted into selected positions within two of the viral proteins, i.e.
25 in exchange for one of the variable antigenic regions located at the surface of hemagglutinin (Muster et al., Mucosal model of immunization against human immunodeficiency virus type 1 with a chimeric influenza virus. J. Virol. 69 (11), 6678-6686 (1995)), or into the stalk sequence of viral neuraminidase (Garcia-Sastre and Palese, The cytoplasmic tail of the
30 neuraminidase protein of influenza A virus does not play an important role in the packaging of this protein into viral envelopes. Virus Res. 37, 37-47 (1995)), and be stably maintained as functional fusion proteins.

Constructs of this kind turned out to be useful for experimental vaccination in a few cases studied, but only rather few clearly defined epitope sequences (of ten to twelve amino acids each) are known today, and some of them might also be misfolded within such restricted fusion protein positions, or in other cases interfere with the correct tertiary structure and function of their host polypeptide chains.

Incorporation of a full-size foreign protein into influenza via reverse genetics, encoded by an independent ninth vRNA molecule in addition to its regular set of eight standard vRNA segments is without special provisions only transiently possible (Luytjes et al., Amplification, expression, and packaging of a foreign gene by influenza virus. Cell 59, 1107-1113 (1989); Enami et al., An influenza virus containing nine different RNA segments. Virology 185, 291-298 (1991)). In the absence of a continuous selective pressure any additional recombinant vRNA segment cannot be stably maintained as long as a wildtype promoter sequence is used on that ninth vRNA segment, and it will inadvertently be lost after few steps of propagation.

Using a different system of influenza reverse genetics developed in our laboratory (Zobel et al., RNA polymerase I catalysed transcription of insert viral cDNA. Nucleic Acids Res. 21, 3607-3614 (1993); Neumann et al., RNA polymerase I-mediated expression of influenza viral RNA molecules. Virology 202, 477-479 (1994)), which was built around *in vivo* synthesis of recombinant vRNA molecules by cellular RNA polymerase I transcription of the respective template cDNA constructs, promoter-up mutations have been designed by nucleotide substitutions (Neumann and Hobom, Mutational analysis of influenza virus promoter elements *in vivo*. J. Gen. Virol. 76, 1709-1717 (1995)). When these are attached to a recombinant ninth vRNA segment its increased transcription and amplification rate will not only compensate for the losses suffered spontaneously, but even cause accumulation of the foreign vRNA segment during simple viral passaging, in the absence of any selection. However, due to its over-replication relative to all of the regular influenza vRNA segments (which of

course are connected to wild-type promoter sequences) after catching up with the others the foreign segment will become over-abundant. This increasingly will result in viral particles that have incorporated several copies of recombinant vRNA, but no longer have a full set of all eight standard segments incorporated among an average of about 15 vRNA molecules present within a virion. Such particles are defective and will not result in plaque formation, hence after an initial increase of recombinant viral particles during the first steps of propagation a dramatic decrease is observed, usually at the third or fourth step of viral passaging, depending on the size of the recombinant vRNA and the level of the promoter-up mutation attached. A balanced situation with regard to the insert length and the level of promoter activity can be achieved, and has been propagated in a particular case over 11 passages, with essentially stable levels of recombinant viruses among a majority of helper viruses (around 80%) during these steps. If a full-level promoter-up mutation is used (1104 or the new variant 1920, see below) a balanced-level propagation is reached in conjunction with a recombinant vRNA size of 4000 nucleotides (Maysa Azzeh, Ph.D. Thesis, Univ. Giessen (2000)).

In all of these preparations, both in transiently achieved increased yields (up to 40% of recombinants after three or four steps of viral passage), and in a balanced propagation of recombinant influenza viruses (10 - 20%) the respective viral progeny inadvertently constitute mixtures with a majority of non-recombinant helper viruses. These result both from a statistical mode of packaging vRNA molecules into a virion (the ninth segment may not be co-packaged), and from the fraction of cells solely infected by helper virus.

The problems of fractional yields and of instability during viral propagation of recombinant influenza are the problems to be solved with the present invention.

Summary of the Invention

Starting out from two observations in this laboratory which are discussed below and which concern two hitherto unsuspected properties of influenza viral RNA polymerase in its interaction with terminally adapted influenza-specific RNA molecules, a new technique for the construction of stable recombinant influenza viruses was found.

As previously described in WO 96/10641 plasmid constructs are designed to generate influenza vRNA-like molecules *in vivo* by cellular RNA polymerase I transcription following plasmid DNA transfection into tissue culture cells, and to this end contain flanking rDNA promoter and terminator elements, externally located relative to any cDNA constructs in between. The resulting recombinant vRNA molecules are designed to contain 5' and 3' recognition sequences for influenza viral RNA polymerase, which however carry up to five nucleotide substitutions (in promoter-up mutant pHL1920) resulting in a substantial increase of expression over wildtype influenza promoter levels. While recombinant pseudoviral RNA is initially transcribed by RNA polymerase I, further amplification and mRNA transcription depends on the presence of viral RNA polymerase and viral nucleoprotein in the cell, which generally are provided by infection of a helper virus. As a consequence the progeny viral yield will constitute a mixture of recombinant viruses together with a majority of wild-type helper viruses.

In the new technique the recombinant vRNA-like molecules as transcribed by RNA polymerase I are constructed as ambisense RNA double segments, with one reading frame (an influenza gene) in sense and a second one (a foreign gene) in anti-sense orientation, or vice versa. In such constructs both reading frames will be expressed via the cap-snatching mode, even if at different levels. Again, infection by helper virus is required to provide the necessary viral early and late proteins for genetic expression and virion packaging. However, the particular helper virus used in the new method is a recombinant virus carrying 2x2 specifically designed ribozyme targets inserted into the flanking non-coding sequences of one of its eight

vRNA segments. The viral segment chosen to become ribozyme-sensitive is always identical to the one used in constructing the recombinant ambisense RNA molecule, as the viral carrier gene in covalent linkage with an additional foreign gene.

- 5 Recombinant influenza viruses produced in this way through RNA polymerase I transcription of an ambisense viral RNA molecule followed by infection with that specifically designed type of ribozyme-sensitive helper virus will carry one of the influenza genes twice, once within that ribozyme-sensitive helper vRNA segment, and a second time within the
- 10 ribozyme-resistant ambisense segment. Recombinant viruses of this type are again obtained initially only as a mixture together with a majority of non-recombinant helper viruses. A progeny viral passage through tissue culture cells (293T) which before have been transiently transfected with plasmid constructs expressing the respective double-headed ribozyme will
- 15 (in one step) inactivate the ribozyme-sensitive segment by a factor of up to 100. One or two rounds of such ribozyme treatment *in vivo* will at the same time (a) purify the recombinant virus from its non-recombinant helper contaminants, and (b) delete the sensitive vRNA helper segment from within the initial (additive) recombinant virus.
- 20 As a result recombinant influenza viruses are isolated along this several-step procedure, which are free of contaminating helper viruses and carry seven regular and one ambisense vRNA segments, all in a balanced replication mode. Their recombinant nature is stably maintained because of a covalent junction between one of the viral genes and the full-size
- 25 foreign gene inserted, a situation achieved here for the first time, via constructing an influenza ambisense RNA segment. The whole procedure is independent of any (selectable) phenotype, and can be applied to either of the eight influenza vRNA segments. After establishing a first ambisense vRNA segment carrying a single foreign gene it can also be repeated all
- 30 over for inserting a second foreign gene within another ambisense RNA segment of the same constitution in principle.

Stable recombinant viruses of the type described can be used for cheap propagation in fertilized eggs, either for production of those recombinant viruses themselves or for production of foreign proteins or glycoproteins encoded by them, and hence find application in (glyco)protein production or in providing vector systems for somatic gene therapy or in being used as vaccination agents.

Thus, the present invention provides

- (1) a recombinant influenza virus for high-yield expression of incorporated foreign gene(s), which is genetically stable in the absence of any helper virus and which comprises at least one viral RNA segment being an ambisense RNA molecule (hereinafter "ambisense RNA segment") and containing one of the standard viral genes in sense orientation and a foreign, recombinant gene in anti-sense orientation, or *vice versa*, in overall convergent arrangement;
- (2) a preferred embodiment of the recombinant influenza virus defined in (1) above, wherein one or more of the regular viral RNA segments, differing from said at least one ambisense RNA segment (hereinafter "modified regular segment"), comprises a vRNA encoding a foreign gene, preferably one or more of the regular viral RNA segments has (have) been exchanged for a vRNA encoding a foreign gene;
- (3) a preferred embodiment of the recombinant influenza virus defined in (1) and (2) above, in which the terminal viral RNA sequences of said one or more modified regular segments and/or of said at least one ambisense RNA segment, which are active as the promoter signal, have been modified by nucleotide substitutions in up to five positions, resulting in improved transcription rates of both the vRNA promoter as well as the cRNA promoter as present in the complementary sequence;
- (4) a method for the production of recombinant influenza viruses as defined in (1) to (3) above comprising
 - (a) RNA polymerase I synthesis of recombinant vRNAs *in vivo*, in antisense, sense or ambisense design,

(b) followed by infection with an influenza carrier strain constructed to include flanking ribozyme target sequences in at least one of its viral RNA segments, and

(c) thereafter selective vRNA inactivation through ribozyme cleavage;

5 (5) a method of constructing influenza carrier strains carrying one or more ribozyme target sites (of type one) in vRNA flanking positions comprising

(a) RNA polymerase I synthesis of recombinant vRNAs *in vivo*, carrying two different 3' promoter sequences in tandem, which are separated by a second type of ribozyme target sequence, and which carry the said

10 internal ribozyme target sites of type one;

(b) followed by infection of an influenza wildtype strain;

(c) thereafter amplification through simple steps of viral propagation; and

(d) finally isolation through removal of their external 3' promoter sequence by ribozyme cleavage through infection of cells expressing

15 ribozyme type 2, followed by plaque purification, said method being suitable for the construction of an influenza carrier strain required for step (b) of (4) above;

(6) a ribozyme-sensitive influenza carrier (helper) strain obtainable by the method of (5) above;

20 (7) a pharmaceutical composition comprising a recombinant influenza virus as defined in (1) to (3) above;

(8) the use of a recombinant influenza virus as defined in (1) to (3) above for preparing a medicament for vaccination purposes;

(9) the use of a recombinant influenza virus as defined in (1) to (3) above
25 for preparing agents for somatic gene therapy;

(10) the use of a recombinant influenza virus as defined in (1) to (3) above for preparing agents for transfer and expression of foreign genes into cells (abortively) infected by such viruses;

(11) the use of a recombinant influenza virus as defined in (1) to (3)
30 above for preparing agents for transfer and expression of RNA molecules into cells infected by such viruses;

(12) a method for the production of proteins or glycoproteins which comprises utilizing a recombinant influenza virus as defined in (1) to (4) above as expression vector;

(13) a method for preventing and/or treating influenza which comprises administering a recombinant influenza virus as defined in (1) to (3) above to the mammal to be treated, i.e., a vaccination method utilizing said recombinant virus;

(14) a method for somatic gene therapy, which method comprises subjecting the organism to be treated with a recombinant influenza virus as defined in (1) to (3) above;

(15) a method for transfer and expression of foreign genes into cells, and for transfer and expression of RNA molecules into cells, which method comprises infecting the cells with a recombinant influenza virus as defined in (1) to (3) above;

(16) use of a recombinant influenza virus as defined in (1) to (3) above for preparing agents for autologous immunotherapy;

(17) a method for an immunotherapy which comprises *ex vivo* infection of immune cells with a recombinant influenza virus as defined in (1) to (3) above, and introduction of the transduced cells into the patient; and

(18) a method for the induction of antibodies which comprises utilizing a recombinant influenza virus as defined in (1) to (3) above as an immunogen.

Brief Description of the Figures

Fig.1 shows 3' nucleotide extensions of influenza vRNA template molecules.

Fig. 2 shows propagation of recombinant influenza viruses with tandem bicistronic vRNA.

Fig. 3 shows tandem bicistronic vRNA supporting an alternative mode of transcription and replication initiation

Fig. 4 shows the outgrowth of promoter-up recombinant vRNA versus wildtype vRNA segments in stepwise propagation of influenza virus.

Fig.5 shows pAM403 ribozyme cleavage of pHL2969 derived vRNA molecules at specific target sites inserted between an external and an internal 3' promoter sequence.

Fig. 6 shows comparative cleavage analysis of model CAT vRNAs with tandem target sites in various flanking positions, by target-specific ribozymes.

Fig. 7 shows an alignment of pAM424 double-headed ribozyme with one of their repetitive target sequences located within the 5' and 3' vRNA flanking regions.

Fig. 8 shows pAM424 ribozyme cleavage of resistant FPV wild-type HA vRNA and ribozyme-sensitive pHL2969-derived HA-vRNA in 293T cells.

Fig. 9 shows a functional analysis of the influenza cRNA promoter structure.

Fig.10 shows a functional analysis of the vRNA and cRNA promoter in ambisense arrangement.

Fig.11 shows a basepair substitution according to the vRNA 'corkscrew' structure.

Fig. 12 shows a flow-chart of the isolation procedure for an ambisense recombinant influenza virus.

Fig. 13 shows immuno-electron microscopy of purified influenza FPV/CSFV-E2-HA virions.

Fig. 14 shows vector pHL2969.

Fig. 15 shows vector pAM403.

Fig. 16 shows vector pAM424.

Fig. 17 shows vector pHL2507.

Fig. 18 shows vector pHL2583.

Fig. 19 shows vector pHL2989.

Fig. 20 shows vector pHL1920.

30 Detailed Description of the Invention

According to the present invention "influenza virus" embraces influenza A virus, influenza B virus and influenza C virus, with influenza A virus being

preferred. A "mammal" according to the present invention includes humans and animals. "Organism" embraces prokariotic and eukariotic systems as well as multicellular systems such as vertebrates (including mammals) and invertebrates. "Infected cells" and "infecting cells" according to the present invention also include "abortively infected cells" and "abortively infecting cells", respectively.

In a preferred influenza virus according to embodiment (1) at least one of the regular viral RNA segments is replaced by an ambisense RNA segment which contains one of the standard viral genes in sense orientation and a foreign, recombinant gene in anti-sense orientation or vice versa in overall convergent arrangement. It is moreover preferred that in the ambisense RNA molecule said foreign recombinant gene is covalently bound to one of the viral genes while the original vRNA segment coding for the same gene is deleted from the recombinant virus by a specific ribozyme cleavage.

The foreign gene(s) in ambisense covalent junction with the viral gene(s) preferably code for proteins and/or glycoproteins which are secreted from cells infected with the recombinant virus, such as lymphokines, or code for glycoproteins that are incorporated into the virion as well as the plasma membrane of the infected cell. In another preferred embodiment the foreign gene(s) in ambisense covalent junction with the viral gene(s) code for proteins or artificial polypeptides designed to support an efficient presentation of inherent epitopes at the surface of infected cells, for stimulation of B cell and/or T cell response. Such proteins or artificial polypeptides constitute for instance a tumor antigen or an artificial oligomeric series of T cell epitopes. Finally, the foreign gene(s) may be suitable for transfer and expression of RNA molecules, including antisense RNAs and double stranded RNAs, into cells. Such recombinant influenza viruses are suitable for sequence specific gene silencing, for example by antisense or RNA interference mechanisms.

A preferred recombinant virus of embodiment (2) of the invention is where in the regular viral RNA segments one or both of the standard glycoproteins hemagglutinin and neuraminidase have been exchanged, preferably into foreign glycoprotein(s) or into fusion glycoproteins consisting of an anchor segment derived from hemagglutinin and an ectodomain obtained from the foreign source, viral or cellular, or in which such recombinant glycoprotein has been inserted as a third molecular species in addition to the remaining standard components.

- 10 As set forth in embodiment (3) above, a preferred recombinant virus of the invention is where the terminal viral RNA sequences, which are active as promoter signal, have been modified by nucleotide substitution in up to 5 positions, resulting in improved transcription rates (of both the vRNA promoter and in the cRNA promoter as present in the complementary sequence) as well as enhanced replication and/or expression rates relative to the wild-type sequence. Said modified terminal viral RNA sequences differ from the wild-type sequence in that they are containing at least one segment (a naturally occurring segment or an additional segment) wherein the 12 nucleotide conserved influenza 3' terminal sequence has been modified by replacement of one to three nucleotides occurring in said sequence at positions 3, 5 and 8 relative to the 3' end by other nucleotides provided that the nucleotides introduced in positions 3 and 8 are forming a base pair (i.e., if the nucleotide position 3 is G, than that in position 8 is C; if the nucleotide in position 3 is C, than that in position 8 is G; etc.).

The 3' conserved regions of the wild-type influenza virus have the following sequences:

Influenza A: 5'-CCUGCUUUUGCU-3'

30 Influenza B: 5'-NN(C/U)GCUUCUGCU-3'

Influenza C: 5'-CCUGCUUCUGCU-3'.

Moreover, the 13 nucleotide conserved influenza 5'-terminal sequence may be modified by replacement of one or two nucleotides occurring in said sequence as positions 3 and 8 by other nucleotides, again provided that the introduced nucleotides are forming a base pair. The 5' conserved regions of the wild-type influenza virus have the following sequences:

Influenza A: 5'-AGUAGAAACAAGG

Influenza B: 5'-AGUAG(A/U)AACA(A/G)NN

Influenza C: 5'-AGCAGUAGCAAG(G/A):

Preferred influenza viruses of the invention are those wherein in the 3' conserved region the replacements G3A and C8U have been performed, more preferred are those where also the replacement U5C has been performed (the above mutations are relative to the 3' end; such counting from the 3' end is also indicated by a line on top of the digit, e.g., G $\overline{3}$ A).

Another preferred influenza virus mutant comprises the 3'-terminal nucleotide sequence G3C, U5C and C8G (relative to the 3' end) giving the following 3' terminal nucleotide sequence 5'-CCUCGUUCUCCU-3'. Among the influenza viruses defined hereinbefore those having a 3'-terminal nucleotide sequence of 5'-CCUGUUUCUACU-3' are most preferred. In case of an influenza A virus the segment may further have the modifications U3A and A8U in its 5' terminal sequence, in case of influenza C it may have the modifications C3U and G8A in its 5' terminal sequence. The most preferred influenza viruses of the present invention comprise the following general structures:

Influenza A (mutant pHL 1104):

5'-AGUAGAAACAAGGNNNU₅₋₆..(880-2300 ntds)..N'N'N'CCUC \overline{U} U \overline{U} C \overline{U} A \overline{C} U-3'

Influenza A (mutant pHL 1920):

5'-AG \overline{A} A \overline{G} AA \overline{U} CAAGGNNNU₅₋₆..(880-2300 ntds)..N'N'N'CCUC \overline{U} U \overline{U} C \overline{U} A \overline{C} U-3'

Influenza A (mutant pHL 1948):

5'-AGUAGAAACAAGGNNNU₅₋₆..(880-2300 ntds)..N'N'N'CCUC \overline{G} U \overline{U} C \overline{U} C \overline{C} U-3'

Influenza B:

5'-AGUAG(A/U)AACA(A/G)NNNNNU₅₋₆..(880-2300 ntds)..N'N'N'N'(C/U)GUUUCUACU-3'

Influenza C:

5'-AGUAGUAACAAG(G/A)GU₅₋₆..(880-2300 ntds)..CCCCUGUUUCUACU-3'

5

In the above structures the variables are defined as follows:

(1) Underlined and enlarged letters show the required mutations relative to the wild-type sequence for preparing a promoter mutant with enhanced properties;

10 (2) enlarged A in the 5'-part of the sequence: additional A (position 10), angle-forming;

(3) (A/G) at one position: different isolates or single segments with different sequence at this respective positions which are analytically interchangeable;

15 (4) N and N': undefined, but base paired positions relative to each other in complementarity between the 5' and 3' termini, different among the 8 segments, but constant for each segment throughout all viral isolates;

(5) (880-2300 ntds): the lengths of the virus segments, in case of segments with foreign genes increased up to 4,000 nucleotides.

20

The pharmaceutical composition according to embodiment (7) above and the medicament of embodiment (8) above contains the recombinant influenza virus in a pharmaceutically effective amount. Besides said recombinant influenza virus, the pharmaceutical composition and the
25 medicament may contain further pharmaceutically acceptable carrier substances well-known to a person skilled in the art, such as binders, desintegrants, diluents, buffers, preservatives, etc. The pharmaceutical composition and medicaments are solid or liquid preparations and are suitable to be administered orally, intravenously or subcutaneously.

30

The medicament according to embodiment (8) above is preferably suitable as a medicament against influenza and/or against other infections. The

recombinant influenza virus may be present in form of inactivated preparations or may be present in form of live recombinant viruses, preferably as attenuated viruses.

- 5 Live recombinant viral vaccines, live but attenuated recombinant viral vaccines or inactivated recombinant viral vaccine can be formulated. Inactivated vaccines are "dead" in the sense that their infectivity has been destroyed. Ideally, the infectivity is destroyed without affecting its immunogenicity. To prepare inactivated vaccines, the recombinant virus
10 may be grown in cell cultures or in embryonated chicken eggs, purified, and inactivated by formaldehyde or β -propiolactone. The resulting vaccine is usually administered intramuscularly.

- Inactivated viruses may be formulated with a suitable adjuvant to
15 enhance the immunological response. Such adjuvants may include, but are not limited to mineral gels, e.g., aluminum hydroxide, surface-active substances such as pluronic polyols, lysolecithin, peptides, oil emulsions, and potential useful human adjuvants such as BCG.

- 20 Many methods may be used to introduce the vaccine formulations above, for example the oral, intradermal, intramuscular, intraperitoneal, subcutaneous, or intranasal routes. Where a live recombinant virus vaccine is used, it may be preferable to introduce the formulation via the natural route of infection for influenza virus.

25

- The medicament according to embodiment (8) above is preferably suitable for prophylactic or therapeutic vaccination, or both, against influenza and other infections. For example, recombinant viruses can be made for use in vaccines against HIV, hepatitis B virus, hepatitis C virus, herpes viruses,
30 papilloma viruses, to name but a few. In one embodiment the recombinant virus contains the genes for surface proteins of the viruses, in another the genes for non-structural or regulatory genes. The

recombinant viruses may be present in form of inactivated preparations or may be present in form of live recombinant viruses, or in live, but attenuated viruses. In an attenuated virus the recombinant virus would go through a single or very few propagation cycle(s) and induce a sufficient
5 level of immune response, but would not cause disease. Such viruses lack one of the essential influenza genes or contain mutations to introduce temperature sensitivity.

The agents of embodiments (9)-(11) of the invention are applicable in ex
10 *vivo* and *in vivo* application schemes. The RNA molecule to be expressed by means of the agent of the embodiment (11) is of an antisense sequence or double strand sequence (in ambisense bidirectional transcription) relative to a target cellular mRNA molecule. In embodiment
(11) the agent is preferably suitable for sequence-specific gene silencing,
15 preferably by antisense RNA or RNA interference mechanisms.

The method for the production of proteins or glycoproteins is preferably performed in cell culture cells or in fertilized chicken cells in accordance with standard techniques within the general knowledge of a person skilled
20 in the art. The proteins or glycoproteins to be expressed are those incorporated into the ambisense construct as defined hereinbefore.

The methods according to embodiments (13) to (15), (17) and (18) of the invention include the administration of an effective amount to the mammal
25 or the administration of a sufficient infective dose of the recombinant virus to the cell system that is used for ex vivo therapy or for in vitro investigations, whereby the amount and dose will be determined by a person skilled in the respective arts or knowledgeable of the desired treatments.

30 The agent of embodiment (16) of the invention is preferably utilized to infect, transfect or transduce patient-derived immune cells. The agent is

suitable for treatment of cancer or chronic viral infections. For this purpose, patient derived immune cells, preferably dendritic cells, are *ex vivo* infected with recombinant influenza viruses expressing, e.g., tumor antigens or viral antigens. The transduced cells are then reintroduced into
5 the patient.

The preferred method for immunotherapy of embodiment (17) of the invention is an autologous immunotherapy, wherein the cells which are *ex vivo* infected are patient-derived and the transduced cells are reintroduced
10 into the patient. The diseases to be treated by this method include cancer and chronic viral infections. For details regarding such treatment see discussion of embodiment (16) above.

The method for inducing antibodies according to embodiment (18) of the invention is suitable for inducing antibodies to foreign proteins including glycoproteins, following the administration of protein or glycoprotein antigens as part of a recombinant influenza virus in an authentic conformation, whereby the virus is purified by gentle procedures based on hemagglutination, and the gene is expressed at high rates in the infected
15 cells.
20

As influenza viruses have a wide host range, recombinant influenza viruses can be used to obtain strong immune responses in, and isolate antibodies from, a wide range of animals, including, but not limited to,
25 fowl, pigs, goats, horses, and mice. Further, influenza viruses adapted to the mouse can be used for the infection of mice, by several routes including the intranasal route. This results in infection of the pharyngeal mucosal cells and results in an additional type of B cell response (e.g., as recognized in the ratio of IgG to IgA). Mice are of particular utility in the
30 induction of immune responses in transgenic mice that have been engineered to express human antibodies. As gentle procedures based on hemagglutination are used to purify influenza viruses, antibodies to

antigens in native conformation can be isolated from the infected mammals.

Further preferred embodiments of the invention are set forth herein
5 below.

A. Construction of influenza helper virus strains carrying ribozyme
target sequences in flanking positions within either of the vRNA
segments

10 A.1: Influenza RNA polymerase will initiate transcription and replication from promoter structures located at internal positions in an RNA molecule, not only from the natural position at both ends of a vRNA molecule:

This is true in particular for promoter-up variants in RNA-internal location
15 due to their enhanced binding affinity for viral polymerase. Not only 3' end extensions are tolerated in RNA-internal promoter recognition (Fig. 1), but also 5' extensions as well as extensions at both ends of the RNA template molecule, containing noncomplementary as well as complementary sequence, i.e. potentially present as a double-stranded extension. Finally,
20 also an extension by way of duplication of the promoter sequence (active or inactivated) leads to mRNA transcription and CAT expression, initiated from the active pair of 5' and 3' promoter halves, irrespective if in external or in internal or even in an oblique localization (active 5' promoter sequence in external, active 3' promoter sequence in internal position, or
25 vice versa). RACE-determination of the resulting 5' and 3' ends of viral mRNA and cRNA, i.e. the products of transcription and replication reactions for several of the extended template vRNA constructs proves an exact recognition and sequence-specific initiation at a position equivalent to regular 3' position $\bar{1}$: all of the various template extensions are lost in
30 every product RNA molecule, most likely after only one round of replication.

A.2: Bicistronic (tandem) vRNA molecules carrying an additional 3' specific promoter sequence in a central position between its two genes can be used for an indirect selection method for recombinant influenza viruses:

The method described is applicable for any foreign gene (e.g. CAT) without a selection potential of its own, if inserted into the distal mRNA position (proximal vRNA position, in anti-sense orientation) behind a carrier gene (e.g. GFP) in the proximal mRNA position, able to serve as a transient selection marker. The carrier gene which is used for selection will get lost spontaneously during further propagation. These constructs are equivalent to a 3' extension of the template vRNA by a full-size gene of 750 nucleotides up to a second 3' promoter sequence, in terminal location. While in the set of experiments shown in Fig. 2 the external 3' promoter sequence is maintained throughout as the same promoter-up variant (1104), the internal 3' promoter sequence has been varied to include a full-level promoter-up variant (pHL2270, containing promoter mutant 1104), a medium-level promoter variant (pHL2350, containing promoter mutant 1948), a wildtype promoter construct (pHL2629), and a construct carrying an unrelated central sequence in an otherwise identical design (pHL2300).

Due to the presence of two 3' promoter sequences in conjunction with a single 5' promoter sequence an alternating interaction between them will constitute one or the other active promoter structure (see Fig. 3). While the external location with an adjacent RNA 3' end may have a structural advantage, this appears to be compensated by the shorter distance in an interaction between the 5' sequence and the central 3' sequence in constituting the internal promoter, such that the competition between the two primarily reflects the various internal 3' promoter allele sequences used, compare Fig. 2B and activity ratios indicated above and below the lanes. Translation of the mRNA-distal gene (CAT) is only observed following an internal initiation at the bicistronic vRNA template, resulting in

a spontaneous deletion of the mRNA-proximal (vRNA-distal) gene, GFP, compare right half of Fig. 3. In complementary analyses GFP fluorescence is observed initially for all of the bicistronic constructs, but gets lost on a faster rate from pHL2270 transfected and influenza infected cells (not shown), and will stay unchanged in pHL2300-treated cells. The indirect selection system based on bicistronic (tandem) molecules as designed here and demonstrated for reporter genes GFP and CAT can be used for any other gene without distinct phenotype upon insertion behind an unrelated carrier gene with properties useful in selection. – In employing that technique an initial phase of (repeated) positive selection for infected cells expressing that proximal trait (e.g. by FACS or by magneto-beads) will be followed by a second phase with negative selection, i.e. against that fraction of infected cells still exposing the same property.

A.3: Isolation of an influenza strain designed to carry 2x2 flanking ribozyme target sequences at the 5' and 3' end of vRNA segment 4 coding for hemagglutinin:

The above scheme for an indirect selection of any foreign recombinant gene behind a proximal carrier gene is further modified by deleting the carrier gene altogether. Instead, both 3' terminal promoter sequences (mutant and wildtype) follow each other at a short distance, separated only by a specific, repetitive ribozyme target sequence, - different from other target sequences to be described further below. The cDNA insert following after the second 3' promoter sequence consists mainly of a regular hemagglutinin (H7) coding sequence, however both the 5' and 3' vRNA terminal regions of the insert carry that other ribozyme target sequence (different from the first target sequence mentioned above) inserted in either location in tandem duplication (pHL2969, see Fig. 14).

Due to a superior replication supported by the promoter-up variant located in 3' external position the recombinant HA segment attached to that promoter sequence will become enriched during the first steps of viral propagation, while the originally dominating HA segment of the helper

virus which is under control of a wildtype promoter sequence is consecutively reduced and finally is no longer detectable among viral progeny. This result is documented by RT-PCR analysis of consecutive viral populations as obtained in that stepwise propagation procedure, see Fig.

5 4.

In the next step the viral lysate is twice passaged via infection of cell culture cells (293T) that before have been DNA-transfected by plasmid pAM403 (Fig. 15). This construct has been specifically designed to express a hammerhead ribozyme with flanking sequences complementary to the repetitive GUC-containing target sequence, as present twice in between the external and internal 3' promoter signals in the recombinant HA vRNA segment, see Fig. 5. In this way the extra promoter sequence is cut off from the finally resulting recombinant HA vRNA. Its promoter-up activity was useful in achieving an initial increase in the concentration of recombinant HA vRNA over wildtype HA vRNA, and in finally excluding the latter from further propagation. However, for the same reason that high activity of the promoter variant will cause instability in the resulting viral progeny, and an effective 'substitution' at this time through ribozyme cleavage by the internally located promoter signal, wild-type or slightly improved, will restore stability to the progeny viruses, with all of their eight vRNA segments now brought back in balance to each other. Due to the ribozyme cleavage site at 26 nucleotides 3' of the wild-type promoter sequence (see Fig. 5), in the initial stage that promoter signal is situated in a vRNA-internal location, extended by a 3' terminal sequence of 26 nucleotides. According to the data presented in Fig. 1 this should cause a transient slight reduction in activity, resulting however in one step in regular viral mRNAs and cRNAs, with any initially remaining extra sequence being lost from the finally resulting recombinant HA vRNA.

Progeny viruses still carrying an external promoter-up sequence (before ribozyme treatment or due to incomplete reaction) will not cause any plaque, due to over-replication of one vRNA segment relative to all others which results in a high load of defective particles. However, progeny

viruses which have lost that external promoter element due to ribozyme cleavage will yield regular plaques due to a balanced mode of replication for all eight wild-type or recombinant vRNA segments. Hence plaque purification is used for isolating a pure influenza viral strain carrying 2x2 ribozyme targets in its recombinant HA vRNA segment, with its termini reduced to the wild-type promoter sequence. The nature of the viral strain isolated has been confirmed in this regard by RT-PCR analysis, see Fig. 4.

The above isolation procedure resulting in influenza viral strains carrying 2x2 flanking ribozyme target sequences has been performed twice for the HA coding segment (segment 4) to obtain two different isolates with regard to the orientation of the ribozyme target sequences. In one of the isolates (vHM41, see SEQ. ID NO: 3) the tandem target sites have been inserted into the HA vRNA non-translated sequence both in 5' and 3' location, while according to the second design that 5' tandem target sequence has been included in an inverted orientation, such that it is now present in the cRNA 3' sequence instead (vHM42).

In another experiment the same procedure was used to isolate an influenza strain carrying 2x2 tandem target sites within the 5' and 3' flanking positions of segment 8 vRNA, i.e. coding for genes NS1 and NS2 (vHM81; see SEQ.ID NO:4). And in principle the same could be done for any other influenza segment, in particular since only the reading frame cDNA sequence has been exchanged from HA to NS, with all of the flanking elements directly responsible for that procedure remaining in place, unchanged.

A.4: Ribozyme cleavage and vRNA segment exchange using ribozyme-sensitive influenza strains as helper viruses:

In an initial model experiment a range of ribozyme type and target site locations was probed in designing a series of CAT reporter gene vRNA constructs (analysed in the presence of a surplus of wildtype helper virus) in 293T cells. While all of the ribozyme constructs adhered to the hammerhead model, with 10 to 12 nucleotides of complementary

sequence flanking on either side of the GUC target site, these ribozyme constructs varied from monomer to dimer to trimer repetitions. Ribozyme containing mRNAs were synthesized *in vivo* via the basic vector plasmid pSV2-*neo*, i.e. using the efficient p_{SVe} RNA polymerase II promoter element for expression, and the SV40 origin signal for plasmid amplification, in a cell line (293T or cos-1) with an incorporated SV40 T antigen gene. In addition the pSV2-*neo* mRNA includes the small, 63 nucleotide intron sequence of the SV40 early mRNA which is supposed to be spliced very slowly, thereby extending the pre-mRNA half-life in the nucleus. Each of the pSV2-*neo*-ribozyme plasmid constructs was transfected into 293T cells. Thereafter, recombinant viruses containing dimer target sites either near one end of the molecule only, or near both ends have been used for infection of the transfected cells. Relative activities of ribozyme constructs versus vRNA target sites have been measured via inactivation of CAT acetyl transfer rates in the cell lysates obtained at 8 h post infection (Fig. 6). The highest activities were observed for dimer ribozymes acting on vRNA molecules carrying 2x2 target sequences on both ends of the molecule, either in vRNA 5' and 3' location, or in vRNA 3' and cRNA 3' location, i.e. with an inversion of the target site sequence at the vRNA 5' end.

Consequently, the two constructs carrying tandem ribozyme double targets within both of their non-translated vRNA flanking sequences have been used in the design of ribozyme-sensitive influenza virus strains as described above, with both variants isolated for segment 4 (HA), and one of them for segment 8 (NS). In complementary correspondence the hammerhead ribozyme plasmid used has also been constructed as a double-headed structure with flanking sequences as shown in Fig. 7 (pAM424; for its detailed structure, see Fig. 16).

The three target site-containing influenza strains isolated as described above have been analysed for their sensitivity against ribozyme cleavage by infection of 293T cells, which had been DNA-transfected 18 h earlier by

ribozyme-producing plasmid pAM424, at DNA-transfection rates between 60 and 70 % (as observed in parallel transfections using GFP-expressing plasmid pAM505). Inactivation rates in these one-step control experiments were between 90% and 99% for all three ribozyme-sensitive strains, in
5 their extent mainly depending upon the actual DNA-transfection rates achieved in individual experiments.

In the next step both isolates of HA-coding ribozyme-sensitive viruses, vHM41 and vHM42, have been used in marker-rescue experiments. Here, 293T cells have been first DNA transfected by HA-variant cDNA construct
10 pHL2507 (see Fig. 17), followed after 18 h by vHM41 or vHM42 virus infections at moi 1. The resulting viral supernatant containing e.g. a mixture of ribozyme-sensitive vHM41 carrier virus and pHL2507/vHM41 recombinant virus is propagated in an intermediate step on MDCK cells, which also results in an increase in the fraction of recombinant viruses.

15 Thereafter the resulting virus-containing supernatant is passaged through 293T (or cos-1) cells, which in advance have been transiently transfected by ribozyme-producing pAM424. As may be concluded from the above experiment (Fig. 6) and shown in Fig. 8 vHM41- or vHM42-derived ribozyme-sensitive HA vRNA segments are expected to be inactivated by
20 pAM424-produced ribozyme down to a remaining level of about 1% to 10% (mainly present within cells that are infected, but not DNA-transfected).

Instead, the substitute HA vRNA which originated from pHL2507 plasmid
25 DNA (ribozyme-resistant) becomes exclusively incorporated into progeny virions. For further purification and viral propagation these have been passaged a second time through 293T cells, again in advance DNA-transfected by pAM424, and after another amplification step on MDCK cells the resulting viral preparations have been used for RT-PCR analysis.

30 The resulting viral populations in these marker rescue experiments only contain HA-vRNA molecules derived from pHL2507, which in their PCR

analyses are of intermediate size relative to wildtype HA-vRNA, and vHM41- or vHM42-derived HA-vRNAs, respectively.

Consequently, a set of ribozyme-sensitive influenza strains with targets inserted individually into every vRNA molecule may be used for such one-
5 step marker rescue experiments in general, i.e. for vRNA segment exchange reactions performed in a directional way for any of the eight influenza vRNA segments, without requirement for a selectable change in phenotype (genetic marker).

10 B. Expression of two gene products from ambisense bicistronic influenza vRNA

B.1: The influenza cRNA promoter is active in antisense viral mRNA transcription according to the cap-snatching mode of initiation:

15 While the vRNA template of influenza virus is known to be active in viral mRNA as well as cRNA synthesis, the cRNA template has been described so far only to produce vRNA molecules, as a second step in viral replication. The potential activity of the cRNA promoter in initiating also viral mRNA transcription has not been analysed or even suspected so far,
20 since no antisense (vRNA) reading frame can be detected in any of the viral RNA segments. Also, no U₅/U₆ template sequence element is present in any of the viral cRNAs in an adjacent position to its 5' promoter structure as is the case for all viral vRNAs. This element is known to serve as a template sequence for mRNA terminal poly-adenylation, in repetitive
25 interaction. However, when both elements are provided through reconstruction of an artificial influenza cRNA segment: a reading frame in opposite orientation (CAT), and a U₆ template element in 5' adjacent location, CAT expression can indeed be observed, see pHL2583 (see Fig. 18) in Fig. 9. Similar to the vRNA promoter the cRNA promoter activity is
30 improved by (the same) promoter-up mutations, which essentially consist of basepair exchanges according to the 'corkscrew' model. This model apparently also holds for the cRNA promoter structure as analysed in a

stepwise manner in Fig. 9. While the cRNA promoter has to be superior over the vRNA promoter in its initiation of replication, since the vRNA/cRNA product ratio was determined to be around 10:1 (Yamanaka et al., *In vivo* analysis of the promoter structure of the influenza virus RNA genome using a transfection system with an engineered RNA. Proc. Natl. Acad. Sci. USA 88, 5369-5373 (1991)), the cRNA promoter is observed to be inferior to the vRNA promoter in its initiation rate of transcription (compare pHL2583 with pHL1844 in Fig. 9), at least for all promoter variants tested so far.

A RACE analysis for determination of the 5' ends in pHL2583 cRNA promoter transcribed mRNAs proved this initiation to occur according to the cap-snatching mode, in complete equivalence to standard vRNA promoter controlled transcription initiation.

B.2: Development of ambisense influenza constructs for consecutive expression of two genes (GFP and CAT) from a single viral RNA:

For a bidirectional transcription and translation of influenza RNA segments the two reporter genes GFP and CAT have been arranged in opposite orientation to each other, and the flanking 5' and 3' promoter sequences (adhering to promoter-up variant 1104) had to be reconstructed to include a U₆ poly-adenylation element in either orientation in a 5' promoter adjacent position. This requirement necessarily resulted in a promoter-adjacent 5'-U₆/3'-A₆ complementary structure, both in the vRNA and cRNA terminal sequence (see Fig. 10), which had to be tested for its promoter activity, in either orientation. Therefore, the convergent pair of reporter genes GFP and CAT has been inserted in both orientations, such that CAT transcription is initiated by the vRNA promoter in one construct (pHL2960), and by the cRNA promoter in the other (pHL2989, Fig. 19), and vice versa for GFP expression from both ambisense constructs. In addition, also the CAT gene only has been inserted in either orientation between the 5' and 3' elements of that ambisense promoter, with CAT transcribed by the vRNA promoter in one case (pHL2959), and by the

cRNA promoter in the other (pHL2957). The whole set of constructs allows for a direct comparison with corresponding reference constructs carrying a regular vRNA promoter (pHL1844) or cRNA promoter structure (pHL2583), i.e. carrying only the 5'-adjacent U₆ sequence element and no 3'-A₆ counterpart. The two groups of constructs also differ in insert size, since a single inserted gene roughly accounts for 750 nucleotides, the convergent set of two genes for 1500 nucleotides, with the distal half of both mRNAs in this case remaining untranslated, a situation unusual for influenza viral mRNAs.

As is demonstrated in Fig. 10B for CAT expression of the various ambisense constructs all of them are able to initiate transcription in both orientations, even if at different levels with regard to their vRNA and cRNA promoter-dependent expressions, and also with regard to the insert lengths and convergent arrangements of the GFP/CAT versus CAT-only constructs. Analysis of the GFP expression rates (not shown) yields complementary results, i.e. again vRNA promoter-controlled GFP expression is superior over cRNA promoter expression of GFP. Therefore, individual ambisense clones either show an asymmetric high expression of GFP and low expression of CAT (pHL2960) or vice versa (pHL2989), depending on their orientation of reading frames with regard to the external vRNA and cRNA promoter. Fig. 10C also demonstrates successful propagation of recombinant viruses containing ambisense RNA molecules, which proves survival through amplification, packaging into virions, and expression of both mRNAs in infected MDCK cells (including besides CAT also GFP expression).

B.3: Construction of a superior promoter-up mutation, pHL1920, to be used for improved rates of cRNA promoter expression in ambisense constructs:

An extended analysis of promoter variants, in particular of complementary double exchanges according to the 'corkscrew' model yielded among others variant pHL1920 (Fig. 20) with CAT activity rates considerably

above (125-130% of) the rates observed for standard promoter-up variant '1104' (as present in pHL1844). The '1920' promoter-up variant consists of altogether 5 nucleotide substitutions relative to the wildtype promoter sequence, both in the 5' promoter element (2), and the 3' promoter element (3). The structure of this variant and the whole set of complementary double exchanges is presented in Fig. 11, together with the respective CAT activity measurements, in vRNA promoter constructs. vRNA promoter-up variants also show similarly improved expression in (ambisense) cRNA constructs, even if at generally lower levels than in vRNA constructs. cRNA promoter-up expression is observed at levels similar or somewhat (2x-5x) above the *wild-type* vRNA promoter rate, while vRNA promoter-up constructs show CAT expression rates increased up to 20 or 25 times the wild-type vRNA promoter level. In either case expression rates also depend on the size of the insert, with promoter activity rates decreasing with increasing lengths of the influenza RNA molecules to be transcribed.

B.4: Influenza recombinant viruses containing a foreign gene (CAT) in covalent ambisense linkage with one of the viral genes (HA, NS1/NS2):

The principle solution in designing stable recombinant viruses based on the new properties observed for influenza transcription and replication signals consists in constructing viruses which contain a foreign gene in covalent linkage with one of the (indispensable) viral genes, in ambisense bicistronic organization. Preferably the viral gene is connected to the cRNA promoter, while vRNA promoter expression is used for expression of the foreign gene at rates considerably above the viral mRNA synthesis. The promoter-up variant chosen for constructing the ambisense RNA segment intends to bring its cRNA promoter expression (approximately) into balance with all other viral gene expression levels, which are controlled by wild-type vRNA promoters located at the termini of the seven ordinary influenza segments; the respective choice has to take into consideration the overall length of the ambisense segment.

Isolation of the ambisense recombinant virus employs an RNA polymerase I-transcribed ambisense cDNA construct, which will give rise *in vivo* to ambisense cRNA-type molecules, see Fig. 12. The plasmid DNA transfection mixture used in this step with 293T cells in addition may or
5 may not contain four 'booster' plasmids which under p_{CMV}-control produce the four early influenza proteins from non-viral mRNAs: NP, plus PB1, PB2, and PA, i.e. the three subunits of viral polymerase (Pleschka et al., A plasmid-based reverse genetics system for influenza A virus J. Virol. 70, 4188-4192 (1996)), which will increase in a pre-amplification step the
10 copy number of that ambisense viral cRNA segment. At 18 h post transfection the 293T cells are infected by a ribozyme-sensitive influenza strain, e.g. vHM41, which will supply (again) early and also late viral RNAs. The resulting supernatant which contains a mixture of vHM41 carrier virus and vHM41-derived ambisense recombinant virus (nine vRNA
15 segments) is then passaged directly or via an intermediate step of amplification on MDCK cells onto 293T cells that have in advance been DNA-transfected by ribozyme-producing pAM424. Here, the ribozyme-sensitive vRNA segment of vHM41 will be cleaved at its 2x2 target sites by pAM424 specific ribozymes. In recombinant viruses the vRNA gene lost in
20 this way is re-supplemented through its presence within the ambisense segment. The virus-containing supernatant is passaged for amplification and further purification through ribozyme treatment a second time on 293T cells which again have been pretreated by pAM424 DNA transfection. Absence of ribozyme-sensitive vRNA, and presence only of ambisense RNA
25 in RT-PCR analysis at this stage allows for further amplification on MDCK cells and a final virus stock preparation on embryonated chicken eggs. CAT assays can be used to analyse for the presence and monitor the activity of this model foreign gene through the various steps of isolation and propagation as well as document technical improvements that might
30 be worked out for one or more of the processive stages.

C. Examples for application of helper-free, stable recombinant influenza viruses

C.1: Incorporation of reporter gene GFP in NS/GFP or HA/GFP ambisense segments:

Recombinant viruses of this type will allow to follow-up on influenza infection instantly and continuously in individual infected cells, which may also be counted or documented by fluorescence photography or FACS sorting. With improved temperature resistance (Siemering et al., Mutations that suppress the thermosensitivity of green fluorescent protein. Curr. Biol. 6, 1653-1663 (1993)) GFP expression becomes visible at 2 h after infection and shows bright fluorescence after 4 h p.i., it will be possible to follow-up on the spread of viral infection by GFP fluorescence. Stable fluorescence in abortively infected cells e. g. observed in *ex vivo* treatment of dendritic cells similarly supports a follow-up on their reincorporation into animals; other genes may be incorporated by ambisense vRNA into dendritic cells in the same way.

C.2: Construction of glycoprotein CSFV-E2 carrying influenza, helper-free:

The glycoprotein E2 of CSF virus has been incorporated previously into influenza both as an HA-anchor fusion protein within the viral envelope, and as an additional, unstable ninth vRNA segment into its genome (Zhou et al.; Membrane-anchored incorporation of a foreign protein in recombinant influenza virions. Virology, 246, 83-94 (1998)). Stabilization is now achieved through an ambisense connection with either of the regular viral RNA segments (NS or HA) which also allowed to reach a level of 100% recombinant viruses instead of an hitherto only 20% (Fig. 13), since all carrier viruses are destroyed through ribozyme action, see Figs. 8 and 12. The helper virus containing preparation has already been used successfully as a vaccine against CSFV infection (antibody titers of 1:40000); the increase achieved in recombinant viruses allows a further

improvement in that regard. Also (cost-effective) propagation in fertilized chicken eggs has become possible due to its stable incorporation of the foreign gene as a covalent ambisense construct.

5 C.3: Construction of hepatitis C glycoprotein-carrying recombinant influenza viruses as a candidate vaccine:

Hepatitis C virus is a close relative of CSF virus (hog cholera virus), and in particular its set of two glycoproteins, small-size E1 and larger-size E2, is closely related in structural detail and presumably also in function to the
10 corresponding CSFV proteins. An incorporation of HCV-E2/HA fusion proteins into influenza viral envelopes has been achieved in analogy to the CSFV-E2/HA incorporation. In addition, incorporation of an anchor-fusion glycoprotein HCV-E1/HA or both together (in NS *and* HA ambisense junctions) allows further variations in constructing an influenza-based
15 vaccine for hepatitis C. In analogy to CSFV-E2, neutralizing antibodies are expected to be directed against particular epitopes of HCV-E2, presented in essentially native conformation at the influenza viral envelope.

20 C.4: Stable incorporation of selected influenza T-cell epitopes in ambisense constructions:

Influenza infection is known to result in both, antibody production against that specific viral strain or indeed its epitopes that are located mainly at the surface of HA, and in an increase of specifically primed cytotoxic T-lymphocytes, stimulated by T-cell epitopes primarily located within the
25 essentially invariable core structure of the NP protein. While the humoral response will result in life-long immunity against *that particular* strain of influenza or its epitope structures, the T-cell response will be lost or severely reduced some time afterwards, such that its specificity against influenza in general will fall below protective levels. One way in trying to
30 increase that level of cellular immunity is to enhance the response or recruitment of influenza-specific CTL cells by increasing the level of T-cell epitopes in the infected cell and hence its presentation on the surface by

MHC-I receptors. This is achieved by combining in an ambisense construct the HA gene and a series of repeated T-cell epitope sequences as present in the influenza NP gene, in a model design specific for the BALB/C mouse MHC-I allele. Here, the promoter-up expression rate is realized (in vRNA promoter-controlled initiation) for expression of the repetitive epitope polypeptide chain. Alternatively or in addition a controlled secretion of an interleukin can be achieved from recombinant influenza-infected cells, upon ambisense incorporation of the respective gene preferably into the NS segment. The interleukin to be chosen for this purpose (IL-12 or other) is selected to enhance the longevity of influenza-specific CTL cells or its conversion into corresponding memory cells. In this way an ambisense vaccine strain against influenza itself is achieved with expected protective capacity against influenza in general.

C.5: Exchange of influenza glycoproteins against foreign viral glycoproteins (VSV-G):

The 'marker rescue' experiment described above (section A.4.), i.e. an exchange of one HA gene (ribozyme-sensitive) for another (ribozyme-resistant) can also be used for an exchange of HA for an entirely different glycoprotein, such as the vesicular stomatitis virus G protein, as long as it is attached to the HA anchor segment. Effective incorporation depends always on that C-terminal tail sequence and its interaction with underlying matrix protein M1, and therefore, all constructions consist of fusion proteins in direct analogy to CSFV-E2. VSV-G with or without a foreign anchor sequence has been shown in several other viruses to be able to substitute for the original glycoprotein and to result in infectious viruses with VSV-G specific host-ranges (e.g. in retroviruses, rabies virus, measles virus). G protein in VSV itself as well as on the surface of foreign viruses is the only glycoprotein required for all of the consecutive steps in infection.

Insertion of VSV-G instead of HA in recombinant influenza viruses leaves the second glycoprotein, neuraminidase, without any function, which then will get lost spontaneously from the recombinant viruses. This will further

increase the capacity for an addition of foreign genes, beyond the gain resulting from an exchange of the larger HA for the smaller VSV-G, which might be used for an addition of ambisense constructs.

- 5 The invention is further illustrated in the accompanying figures.

Detailed Description of the Figures

Fig.1: 3' nucleotide extensions of influenza vRNA template molecules:

- 10 (A) Murine B82 cells have been transfected by plasmid cDNA constructs designed to be transcribed into influenza vRNA molecules by RNA polymerase I *in vivo*, followed after 20 h by standard FPV_{Bratislava} helper virus infection, at an moi of 1 to 3. In addition to reference plasmid pHL2024 (no extension), related cDNA constructs carrying extensions of 1
15 to 50 bp, and hence extended by 1 to 50 nucleotides at the resulting vRNA 3' ends were used in parallel transfections; template extensions are marked at the top of the figure. Cell lysates prepared at 8 h post helper virus infections were used for CAT reactions using in one round 50 µl of cell lysate each, and in further analyses 5µl and 0.5 µl of lysate (not
20 shown). Relative yields were determined in comparison to reference plasmid pHL2024, as indicated below the figure, with calculations restricted to those CAT assays showing less than 40% of substrate consumption, in three or more independent experiments.

- (B) Viral passage of B82 supernatants containing recombinant influenza
25 virus onto MDCK cells, at an moi of 2 to 4, in average. Again at 8 h post infection cell lysates have been prepared and used for CAT assays. Relative yields as indicated below the figure have been determined in comparison to pHL2024 the same way as in (A), using 0.5 µl of cell lysate in each case. (The 50 µl CAT assays as shown here and also in the
30 following figures intend to give an immediate impression of relative activities at always the same level, while the actual measurement data as

indicated below the lanes are obtained at various appropriate enzyme concentrations relative to reference pHL2024.)

Fig. 2: Propagation of recombinant influenza viruses with tandem bicistronic vRNA:

(A) General design of expression plasmids for transient bicistronic vRNAs coding for GFP in the mRNA-proximal, and for CAT in the mRNA-distal position. Among the functional elements indicated are the human RNA polymerase I promoter (p_{IH}) and murine rDNA terminator (t_I) sequences, both hatched, and the 5' and 3' vRNA promoter cDNA sequences, open and closed boxes, respectively. For the vRNA-internal 3' promoter signal three variant sequences have been inserted as indicated below (positions 1 to 15 refer to 3'-terminal nucleotides in the resulting monocistronic vRNAs).

(B) CAT assays as determined relative to pHL1844 (monocistronic CAT construct) after DNA transfection of 293T cells plus helper virus infection followed by one round of progeny viral propagation on MDCK cells are indicated *below* the lanes. Relative activities of the internal promoter sequences as indicated *above* the figure refer to measurements in a monocistronic *external* location of the same promoter variants (Flick *et al.*, Promoter elements in the influenza vRNA terminal structure, RNA 2, 1046-1057 (1996)). Control clone pHL2300 contains an unrelated, non-functional sequence in the central location in an otherwise identical plasmid construct.

Fig. 3: Tandem bicistronic vRNA sup-porting an alternative mode of transcription and replication initiation:

An additional internal 3' promoter sequence has been inserted in between both cistrons, in a vRNA-central position. Left half: bicistronic replication and transcription leading to (proximal) GFP expression. Right half: internal initiation resulting in monocistronic replication and transcription leading to

(distal) CAT expression, and causing deletion of the GFP sequence from progeny molecules.

Fig. 4: Outgrowth of promoter-up recombinant vRNA versus wildtype vRNA segments in stepwise propagation of influenza virus:

RNA polymerase I transcription of transfected pHL2969 DNA results in influenza vRNA carrying (an external) promoter-up mutant '1104', and containing 2x2 ribozyme targets in flanking positions relative to its HA coding sequence. Another HA vRNA segment in wild-type configuration and originating from infecting FPV helper virus is also present in the recombinant virus preparation, initially (lane 1; 293T lysate) in surplus amounts, but reduced and finally lost entirely in consecutive steps of propagation (lanes 2 to 4; MDCK cell lysates), and in isolated strains after pAM403 ribozyme treatment for removal of the external '1104' promoter sequence (lane 5). Determination throughout by RT-PCR analyses using a pair of primers extending across the 5' inserted target site sequence, with 435 bp representing the recombinant HA segment, and 306 bp the wild-type sequence without an inserted target site sequence.

Fig.5: pAM403 ribozyme cleavage of pHL2969 derived vRNA molecules at specific target sites inserted between an external and an internal 3' promoter sequence:

The external promoter-up ('1104') signal is used for vRNA amplification within recombinant viruses and reduction of helper virus HA vRNA (Fig. 4), while the 'switch' to an internal wild-type signal guarantees stable replication of recombinant viruses. pAM403 hammerhead ribozyme RNAs are indicated in complementary binding to their target site sequences (12 and 10 nucleotides flanking the GU'C cleavage point) by straight lines flanking a central secondary structure symbol. vRNA-internal 2x2 ribozyme targets are marked by xx (see Fig. 7).

Fig. 6: Comparative cleavage analysis of model CAT vRNAs with tandem target sites in various flanking positions, by target-specific ribozymes:

293T tissue culture cells have been transiently DNA-transfected either by a single-headed hammerhead ribozyme (**s**), or a double-headed (**d**), or triple-headed (**t**) ribozyme cDNA construct, all specifically designed to hybridize to a tandem dimer target site sequence inserted in flanking positions into the CAT vRNA. All ribozyme RNAs have been expressed from the same pSV2-*neo* plasmid vector, including a pSV2-*neo* control construct without an inserted ribozyme cDNA sequence (**c**). At 20 h after DNA transfection (which reached 65% yield as measured by p_{CMV}-GFP transfection in parallel of the same cell culture) the 293T cells were infected by CAT recombinant viruses carrying tandem double target sequences either only in vRNA-3' position, or in both vRNA-3' and 5' positions, or in both vRNA-3' and cRNA-3' positions. Most effective among the *s*, *d*, or *t*-ribozymes were double-headed constructs, acting on 2x2 targets inserted in either of the two localizations described (lanes 6 and 10).

Fig. 7: Alignment of pAM424 double-headed ribozyme with one of their repetitive target sequences located within the 5' and 3' vRNA flanking regions:

The superior activity of ribozymes oriented against targets located in the 3' end of vRNA molecules over those present in the 5' end instead (not shown) is in agreement with the model for influenza vRNA transcription and replication (Lamb and Krug, Orthomyxoviridae: The viruses and their replication. In 'Virology' (B.N.Fields, D.M.Knipe, P.M.Howley, R.M.Chanock, J.L.Melnick, T.P.Monath, B.Roizman, and S.E.Straus, Eds.), 3rd ed., Vol. 1, pp. 1353-1395. Lippincott-Raven, Philadelphia (1996)), according to which influenza polymerase stays attached to the 5' end of the vRNA molecule throughout the entire or even several rounds of transcription, whereas the very 3' end repeatedly, in every initiation reaction serves as the template sequence, and consequently is no longer covered by polymerase.

Superiority of a double-headed over a single-headed ribozyme has been determined earlier in this laboratory (A.Menke, Anti-Influenza Ribozyme: vRNA-Spaltung und intrazelluläre Aktivität. Dissertation Universität Giessen (1997)), but the substantial increase of vRNA inactivation rates upon incorporation of tandem target sites at both ends of the vRNA molecule instead of only one has been observed here for the first time, within that overall design.

Fig. 8: pAM424 ribozyme cleavage of resistant FPV wild-type HA vRNA and ribozyme-sensitive pHL2969-derived HA-vRNA in 293T cells infected by vHM41 after isolation from pHL2969-recombinant viral preparations. Lane 1: FPV infection of 293T cells, untreated; lane 2: FPV infection of 293T cells DNA-transfected by pAM424; lane 3: vHM41 infection of 293T cells, untreated; lane 4: vHM41 infection of 293T cells DNA-transfected by pAM424. RT-PCR analyses of purified viral progeny as in Fig. 4.

Fig. 9: Functional analysis of the influenza cRNA promoter structure:

(A) Schematic cRNA promoter ('1104') secondary structure according to the 'corkscrew' model; nucleotides involved in single or double nucleotide exchange are marked by their position.

(B) CAT analyses of 293T cell lysates after DNA transfection and FPV helper virus infection of cRNA promoter variants, in comparison to standard vRNA promoter-up mutant '1104' (pHL1844). Nucleotide substitutions divergent from the basic '1104' structure as present in pHL2583 or pHL2721 (see above) are indicated above the lanes, positions $\bar{3}$ or $\bar{8}$ as marked by a bar refer to cRNA positions counted from the 3' end. Relative CAT activities are marked below the lanes.

Fig.10: Functional analysis of the vRNA and cRNA promoter in ambisense arrangement:

(A) Sequence organisation of the ambisense promoter cDNA construct carrying T₆/A₆ elements adjacent to the terminal sequence, and secondary structure predictions for the resulting cRNA and vRNA promoter signal.

(B) CAT expression data obtained from the cell lysates of 293T cell after plasmid DNA transfection and FPV infection, and (C) from cell lysates of MDCK cells after one step of viral passage. Indicated above the lanes are promoter/gene conjunctions: v = vRNA promoter; c = cRNA promoter.

Fig.11: Basepair substitutions according to the vRNA 'corkscrew' structure:

(A) 'Corkscrew' conformation of the vRNA promoter drawn against a schematic indication of interacting tripartite viral polymerase. Paired positions exchanged in individual experiments are indicated by numbers, nucleotides $\bar{3}$ or $\bar{8}$ are counted from the 3' end. pHL2024 containing promoter-up mutation '1104' is used as the reference construct (=100%) in all of the CAT assays, while pHL2428 represents the wild-type promoter structure.

(B) CAT expression data obtained after one step of viral passage in MDCK undiluted, and 50 fold diluted.

Fig. 12: Flow-chart of the isolation procedure for an ambisense recombinant influenza virus.

Fig. 13: Immuno-electron microscopy of purified influenza FPV/CSFV-E2-HA virions:

Recombinant viruses exposing the foreign glycoprotein CSFV-E2 in their envelopes, which has been fused onto the HA anchor domain, are marked by anti-E2 monospecific antibody and by secondary gold-labelled (5nm) goat antibody. Recombinant viruses (16%) are present together with their FPV helper viruses.

Fig. 14: pHL2969; the exact sequence of the 4930 bps circular DNA is shown in SEQ. ID NO:1.

Fig. 15: pAM403; the exact sequence of the 5811 bps circular DNA is shown in SEQ. ID NO:2.

5 Fig. 16: pAM424; the exact sequence of the 5860 bps circular DNA is shown in SEQ. ID NO:5.

Fig. 17: pHL2507; the exact sequence of the 4610 bps circular DNA is shown in SEQ. ID NO:6.

10

Fig. 18: pHL2583; the exact sequence of the 3558 bps circular DNA is shown in SEQ. ID NO:7.

15 Fig. 19: pHL2989; the exact sequence of the 4343 bps circular DNA is shown in SEQ. ID NO:8.

Fig. 20: pHL1920; the exact sequence of the 3888 bps circular DNA is shown in SEQ. ID NO:9.

20 Hence, the present invention is based on two surprising findings, namely
1. influenza virus promoters are active when present internally in a gene;
2. the so-called cRNA, thought to be an intermediate in replication can be turned into a protein-encoding RNA by equipping it with a variant influenza virus promoter, described in the present invention.

25

These two observations were used to make ambisense constructs. This allows to package an additional, foreign gene into influenza virus particles. Such particles were made previously, by other methods, but proved to be unstable, and therefore useless. For use as a vaccine for example, a
30 helper virus would have been needed as a stabilizer. Stabilization in the present invention is achieved by several means. These include the "balancing" of one of the two promoters in the ambisense biscistronic

genetic construct with seven other vRNA wildtype promoters, while the additional promoter is used for high-rate expression of the foreign gene at various levels.

- 5 Thus, the present invention provides a system for expression of foreign proteins in higher eukaryotic systems. One system in particular is interesting, namely embryonated chicken eggs, as it allows cost-effective production in an automatable way (as used by most flu vaccine producers). The reason that this process is now possible, is that the
10 foreign protein is part of a stable, engineered influenza virus particle. The virus can be designed also to rapidly monitor process improvements.

- An excellent use is of course the use of the construct as a vaccine. The influenza virus particle is immunogenic and can now be equipped with
15 foreign antigens, enabling for example the design and production of hepatitis C virus and HIV vaccines, but also of tumor vaccines. As the present invention shows, the foreign antigenic surface glycoprotein is "fused" to a C-terminal segment of influenza HA, and the antigen then is presented at the surface of influenza virus particles. In addition, these
20 vaccines can now be made in the way standard flu vaccines are made, i.e., in embryonated chicken eggs.

PCT/EP00/01903

JH/ml

Artemis Pharmaceuticals GmbH

21 May 2001

Claims

1. A recombinant influenza virus for high-yield expression of incorporated foreign gene(s), which is genetically stable in the absence of any helper virus and which has eight viral RNA segments, wherein at least one of the regular viral RNA segments is replaced by an RNA molecule (ambisense RNA segment), said ambisense RNA segment containing one of the standard viral genes in sense orientation and a foreign, recombinant gene in anti-sense orientation, or *vice versa*, covalently linked to each other and in overall convergent arrangement.
2. The recombinant virus according to claim 1, wherein in the ambisense RNA molecule said foreign recombinant gene is covalently bound to one of the viral genes, while the original vRNA segment coding for the same gene is deleted from the recombinant virus by way of specific ribozyme cleavage.
3. The recombinant influenza virus according to claims 1 and 2, wherein one or more of the regular viral RNA segments, differing from said at least one ambisense RNA segment, comprises a vRNA encoding a foreign gene, preferably one or more of the regular viral RNA segments has (have) been exchanged for a vRNA encoding a foreign gene.
4. The recombinant influenza virus according to claim 3 in which one or both of the standard glycoproteins hemagglutinin and neuraminidase have been exchanged into foreign glycoprotein(s) or into fusion glycoproteins consisting of an anchor segment derived from hemagglutinin and an ectodomain obtained from the foreign source, viral or cellular, or in which such recombinant glycoprotein has been inserted as a third molecular species in addition to the remaining standard components.

5. The recombinant influenza virus according to claims 1 to 4, in which the terminal viral RNA sequences of one or more of the regular segments and/or of the at least one ambisense RNA segment, which are active as the promoter signal, have been modified by nucleotide substitutions in up to five positions, resulting in improved transcription rates of both the vRNA promoter as well as the cRNA promoter as present in the complementary sequence.
6. The recombinant influenza virus of claim 5, wherein the 12 nucleotide conserved influenza 3' terminal sequence has been modified by replacement of one to three nucleotides occurring in said sequence at positions 3, 5 and 8 relative to the 3' end by other nucleotides, and/or wherein the 13 nucleotide conserved influenza 5' terminal sequence has been modified by replacement of one or two nucleotides occurring in said sequence at positions 3 and 8 by other nucleotides.
7. The recombinant influenza virus of claim 6, wherein the replacements in the 3' terminal nucleotide sequence comprises the modifications G3A and C8U.
8. The recombinant influenza virus of claim 7, wherein the replacements in the 3' terminal nucleotide sequence comprises the modifications G3A, U5C and C8U, or G3C, U5C and C8G.
9. The recombinant influenza virus of claim 8, which comprises a 3' terminal nucleotide sequence of 5'-CCUGUUUCUACU-3'.
10. The recombinant influenza virus of claims 6 to 9, wherein the 5' terminal nucleotide sequence comprises the modifications U3A and A8U resulting in a 5'-terminal sequence of 5'-AGAAGAAUCAAGG.

11. The recombinant influenza virus according to claims 1 to 10, which is a recombinant influenza A virus.

12. The recombinant influenza virus according to claims 1 to 11, in which the foreign gene(s) in ambisense covalent junction with viral gene(s) code for proteins and/or glycoproteins which are secreted from cells infected with the recombinant virus.

13. The recombinant virus according to claims 1 to 11, in which the foreign gene(s) in ambisense covalent junction with viral gene(s) code for proteins or artificial polypeptides designed to support an efficient presentation of inherent epitopes at the surface of infected cells, for stimulation of a B cell and/or T cell response.

14. A method for the production of recombinant influenza viruses as defined in claims 1 to 13 comprising

(a) RNA polymerase I synthesis of recombinant vRNAs *in vivo*, in ambisense design,

(b) followed by infection with an influenza carrier strain constructed to include flanking ribozyme target sequences in at least one of its viral RNA segments which is (are) to be replaced by the ambisense segments of step (a), and

(c) thereafter selective vRNA inactivation through ribozyme cleavage.

15. A pharmaceutical composition comprising a recombinant influenza virus according to claims 1 to 13.

16. Use of a recombinant influenza virus according to claims 1 to 13 for preparing a medicament for vaccination purposes.

17. The use according to claim 16, wherein the medicament

(a) is suitable against influenza and/or against other infections;

- (b) is present in form of inactivated preparations; and/or
- (c) is present in form of live recombinant viruses.

18. Use of a recombinant influenza virus according to claims 1 to 13 for preparing agents for somatic gene therapy.

19. Use of a recombinant influenza virus according to claims 1 to 13 for preparing agents, for transfer and expression of foreign genes into cells infected by such viruses.

20. Use of a recombinant influenza virus according to claims 1 to 13 for preparing agents for transfer and expression of RNA molecules into cells infected by such viruses.

21. The use of claim 20, wherein the RNA molecules to be expressed are antisense sequences or double-strand sequences relative to the target cell cellular mRNA molecules, and/or the agent is suitable for sequence-specific gene silencing, preferably by antisense RNA or RNA interference mechanisms.

22. The use according to claims 18 to 21, wherein the agents are applicable in *ex vivo* and *in vivo* application schemes.

23. A method for the production of proteins or glycoproteins which comprises utilizing a recombinant influenza virus according to claims 1 to 13 as expression vector.

24. The method of claim 23, wherein the production is performed in cell culture cells or in fertilized chicken eggs.

25. A method for preventing and/or treating influenza which comprises administering an effective amount of a recombinant influenza virus according to claims 1 to 13 to the mammal to be treated.

26. A method for somatic gene therapy, which method comprises subjecting the organism to be treated with a recombinant influenza virus according to claims 1 to 13.

27. A method for transfer and expression of foreign genes into cells, and for transfer and expression of RNA molecules into cells, which method comprises infecting the cells with a recombinant influenza virus according to claims 1 to 13.

28. Use of a recombinant influenza virus according to claims 1 to 13 for preparing agents for autologous immunotherapy.

29. A method for an immunotherapy which comprises *ex vivo* infection of immune cells with a recombinant influenza virus according to claims 1 to 13, and introduction of the transduced cells into the patient.

30. A method for the induction of antibodies which comprises utilizing a recombinant influenza virus according to claims 1 to 13 as an immunogen.

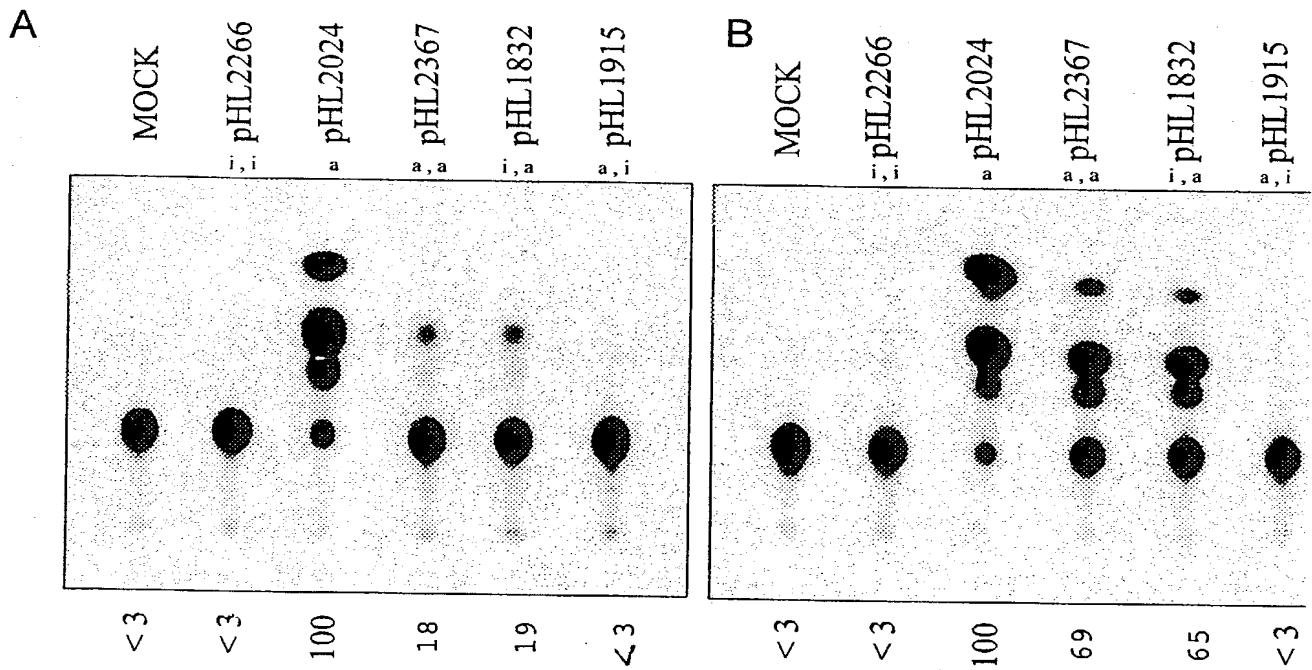
PCT

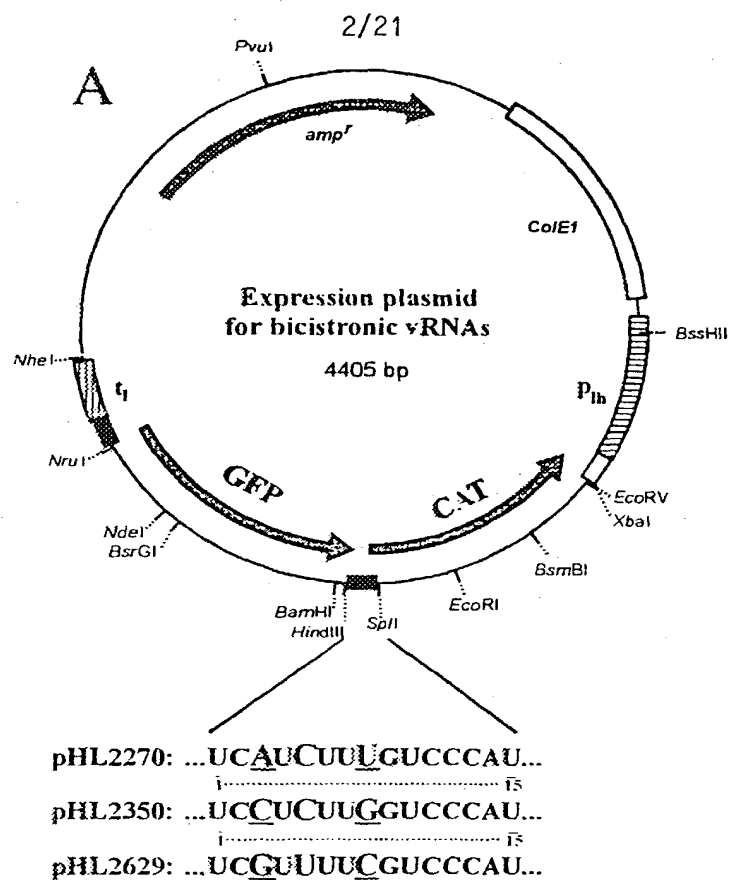
WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

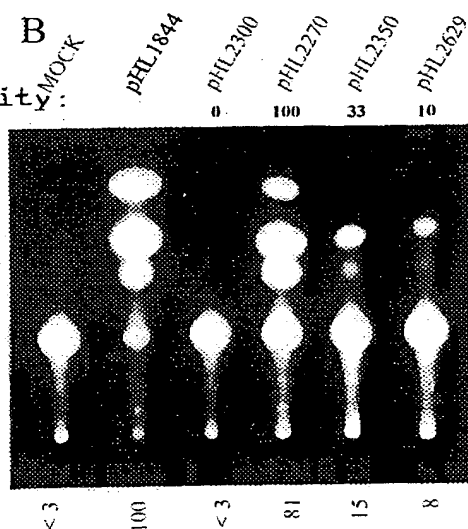
(51) International Patent Classification ⁷ : C12N 15/86, 7/01, 5/10, A61K 39/00, 39/145, 48/00	A1	(11) International Publication Number: WO 00/53786 (43) International Publication Date: 14 September 2000 (14.09.00)
(21) International Application Number: PCT/EP00/01903 (22) International Filing Date: 3 March 2000 (03.03.00) (30) Priority Data: 99104519.6 6 March 1999 (06.03.99) EP (71) Applicant (for all designated States except US): ARTEMIS PHARMACEUTICALS GMBH [DE/DE]; Neurather Ring 1, D-51063 Köln (DE). (72) Inventors; and (75) Inventors/Applicants (for US only): HOBOM, Gerd [DE/DE]; Arndtstrasse 14, D-35392 Giessen (DE). FLICK, Ramon [DE/SE]; Lilla Hallsatrvägen 28, S-137 93 Västerhaninge (SE). MENKE, Anette [DE/DE]; Ockershäuser Allee 7, D-35037 Marburg (DE). AZZEH, Maysa [IL/DE]; Altenfeldsweg 32, D-35394 Giessen (DE). (74) Agents: HELBING, Jörg et al.; Von Kreisler Selting Werner, Deichmannhaus am Dom, D-50667 Köln (DE).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: STABLE RECOMBINANT INFLUENZA VIRUSES FREE OF HELPER VIRUSES		
(57) Abstract <p>The invention relates to a recombinant influenza virus for high-yield expression of incorporated foreign gene(s), which is genetically stable in the absence of any helper virus and which comprises at least one viral RNA segment being an ambisense RNA molecule (ambisense RNA segment) and containing one of the standard viral genes in sense orientation and a foreign, recombinant gene in anti-sense orientation, or <i>vice versa</i>, in overall convergent arrangement. The present invention further provides a method for the production of said recombinant influenza virus, a method for constructing specific ribozyme-sensitive influenza carrier strains; pharmaceutical compositions comprising said recombinant influenza virus; and the use of said recombinant influenza virus for preparing medicaments for vaccination purposes.</p>		

Fig. 1





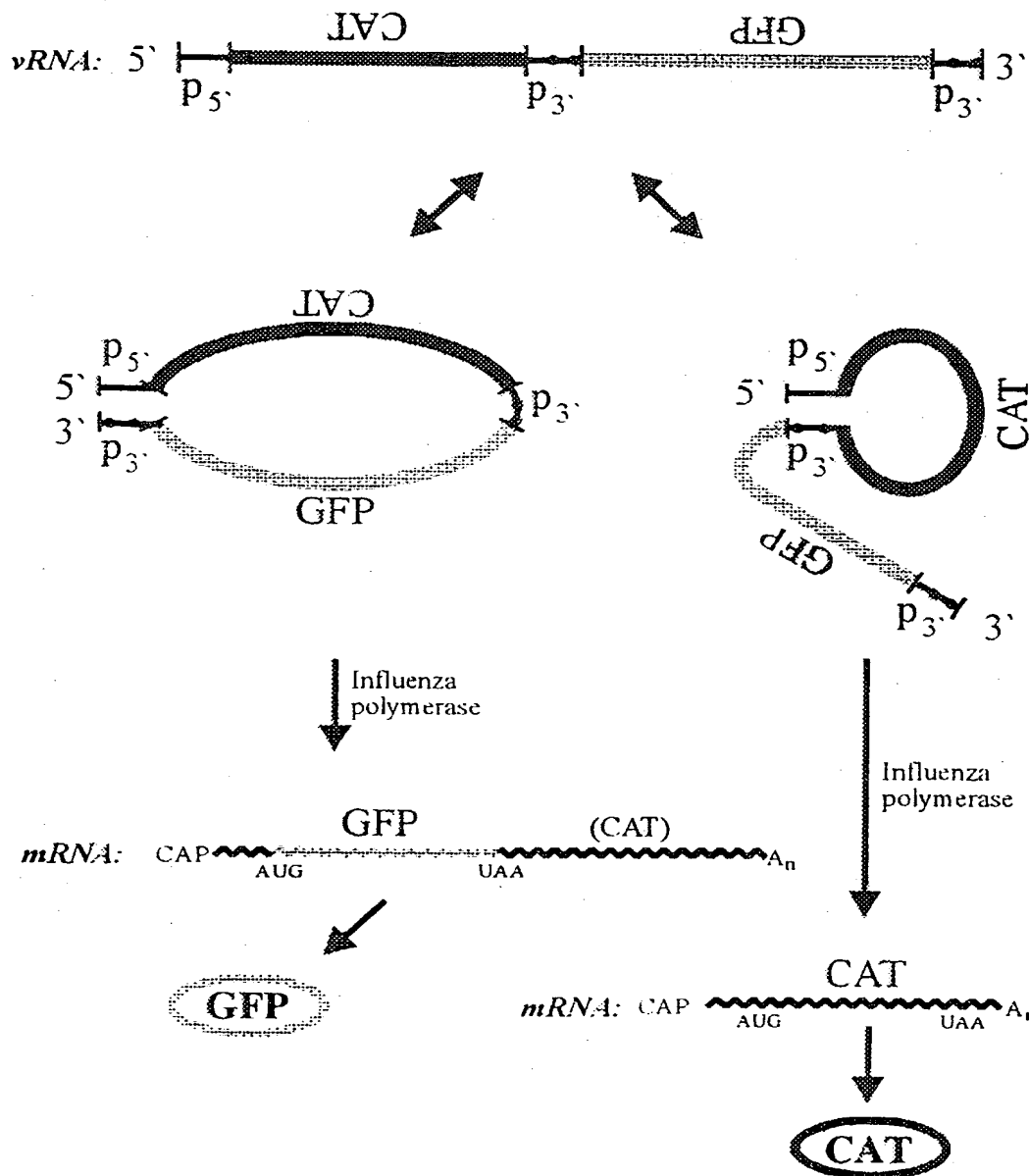
Internal promoter activity:



WO 00/53786

PCT/EP00/01903

3/21
FIG. 3

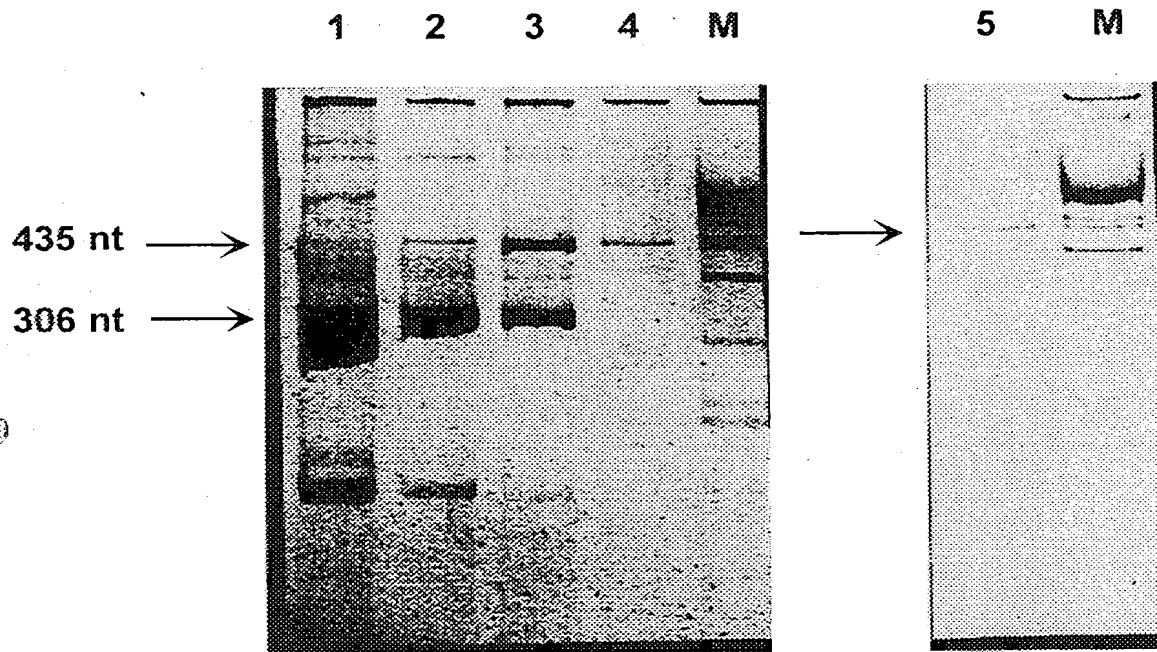


WO 00/53786

4/21

PCT/EP00/01903

FIG. 4

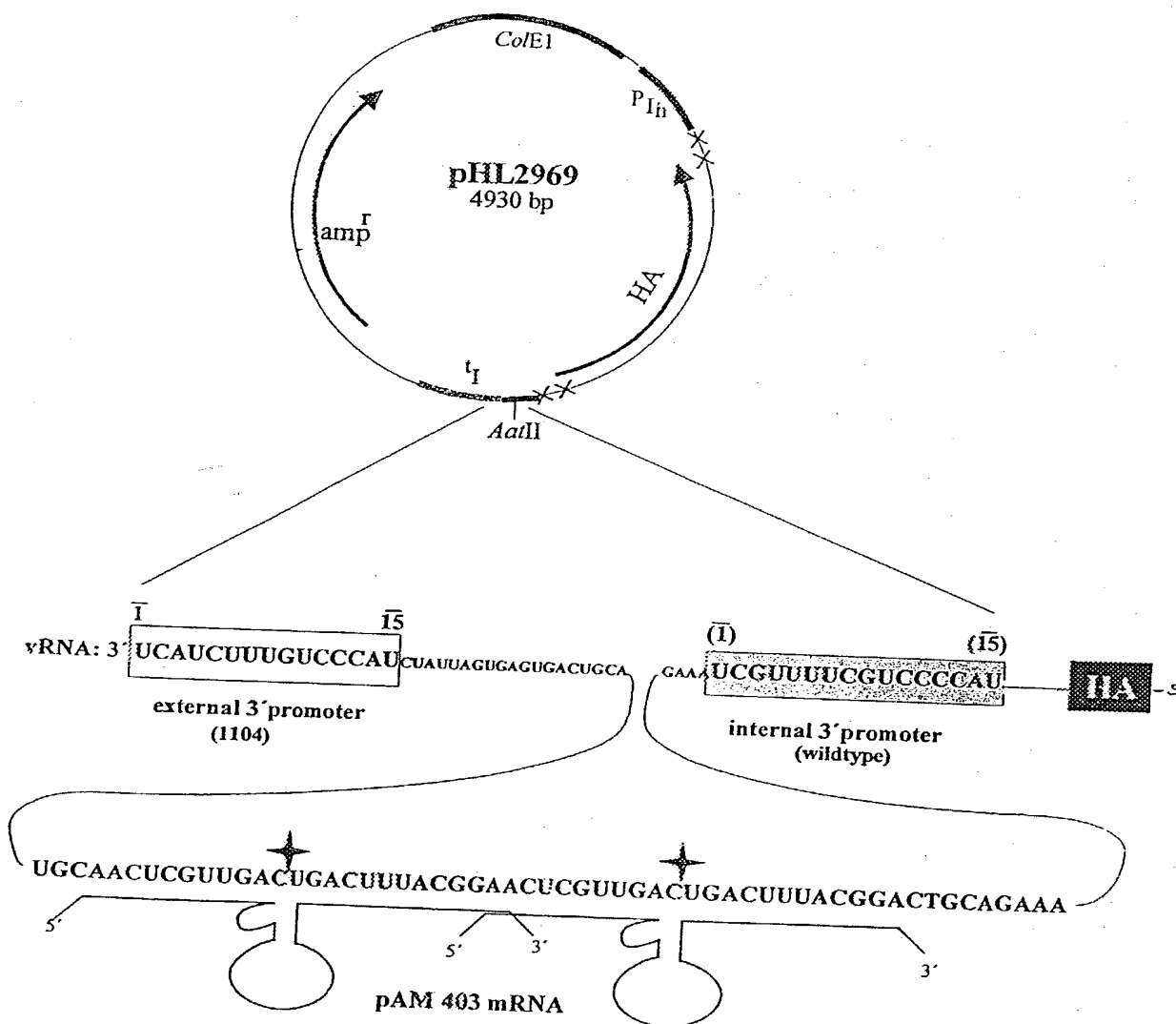


WO 00/53786

PCT/EP00/01903

5/21

FIG. 5



WO 00/53786

6/21

PCT/EP00/01903

FIG. 6

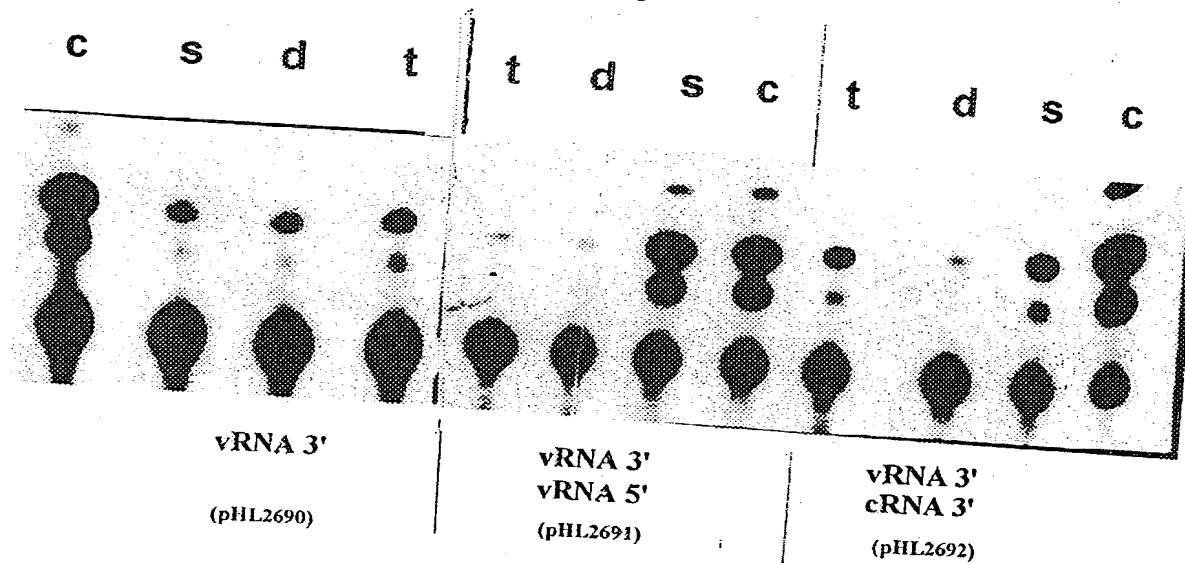


FIG. 7

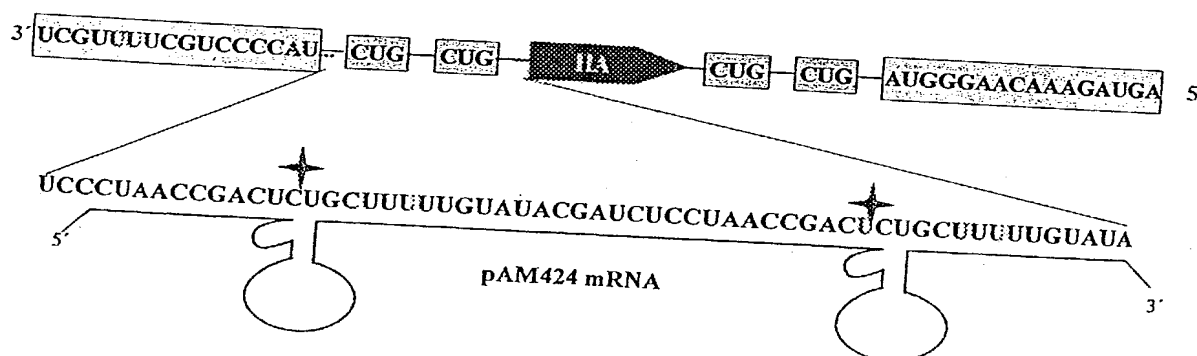


FIG. 8

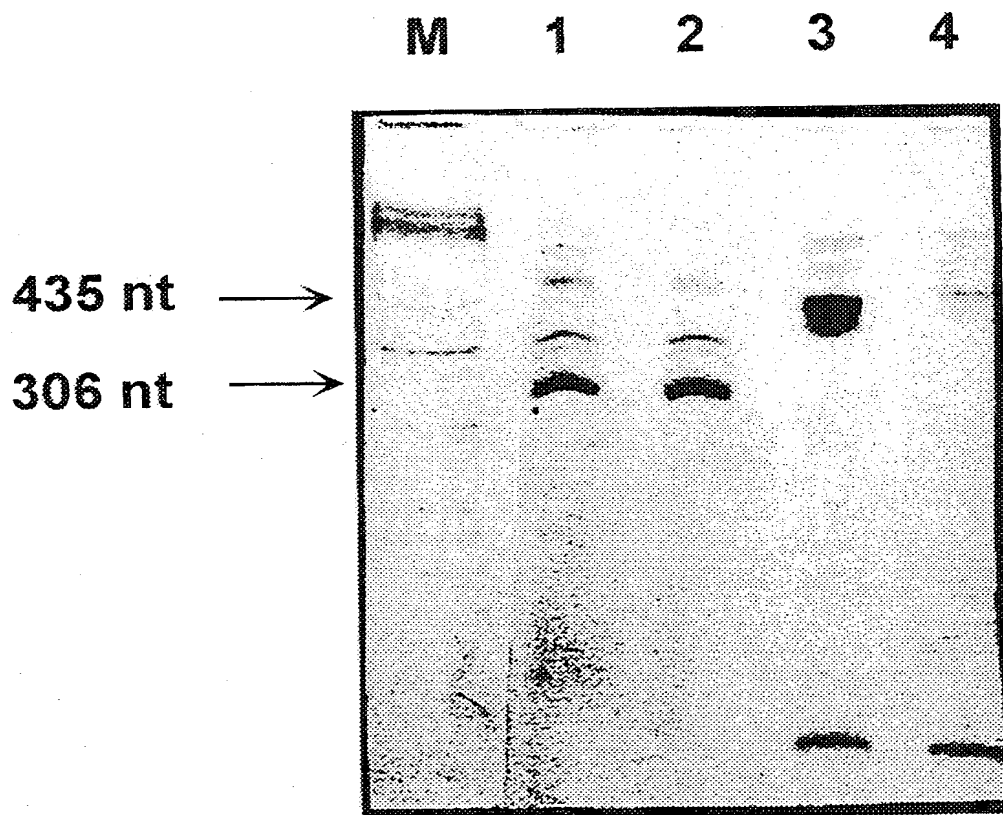


FIG.9

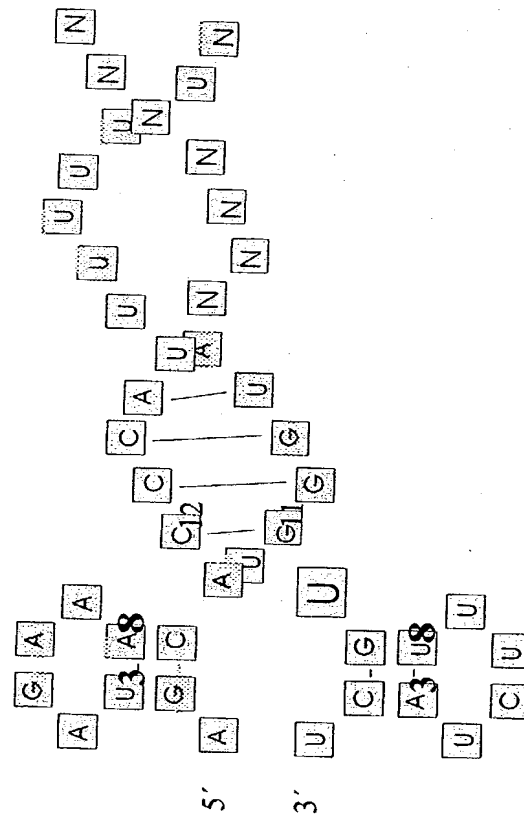


Fig. 9 (continued)

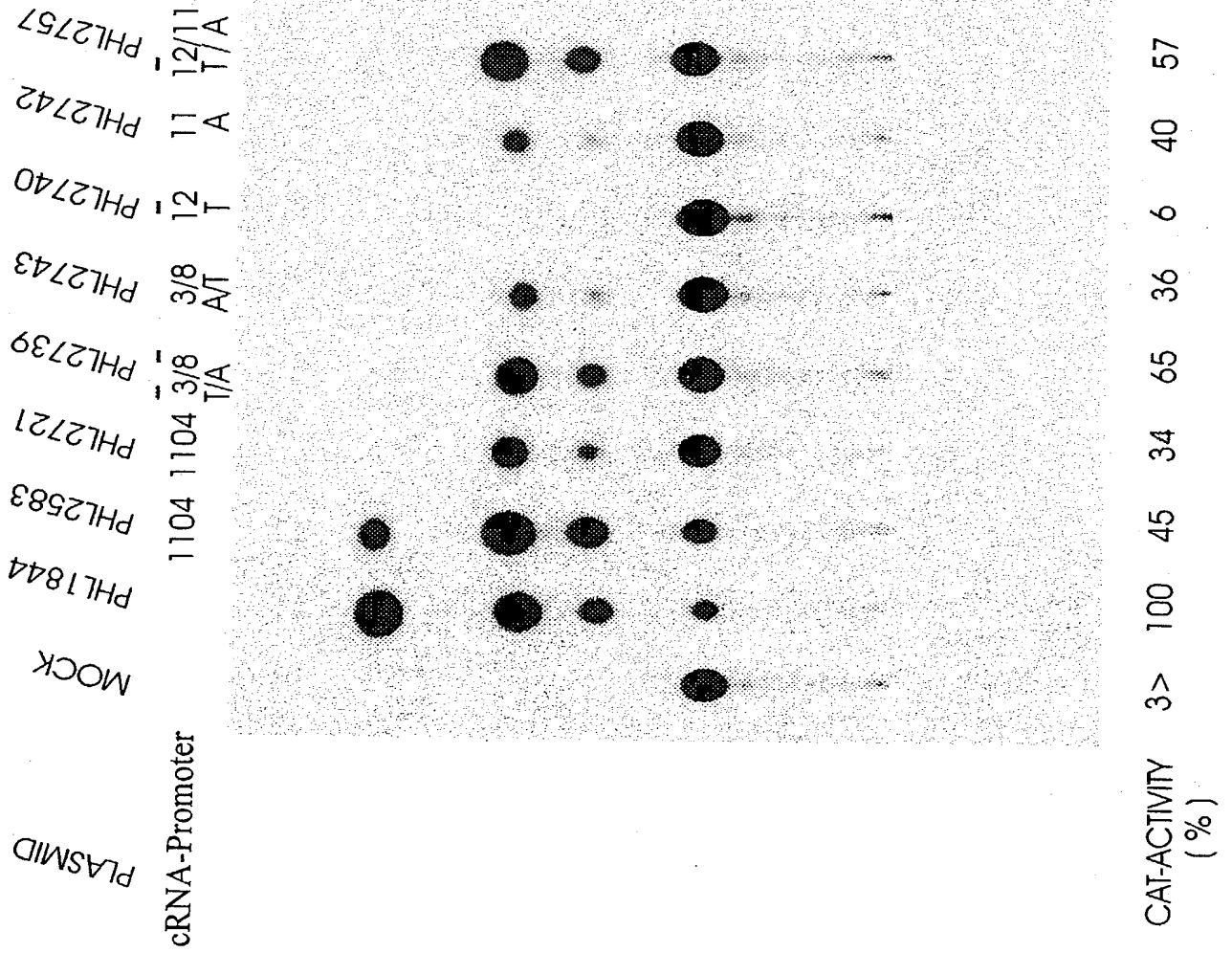
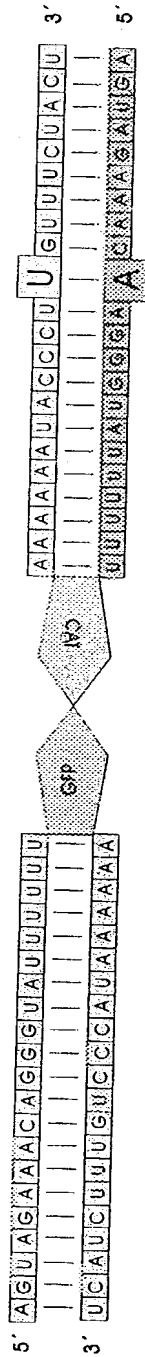
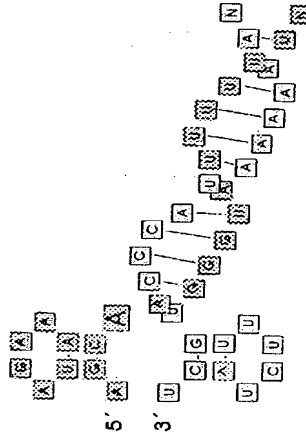


FIG. 10



vRNA- Ambisense Promoter



cRNA-Ambisense Promoter

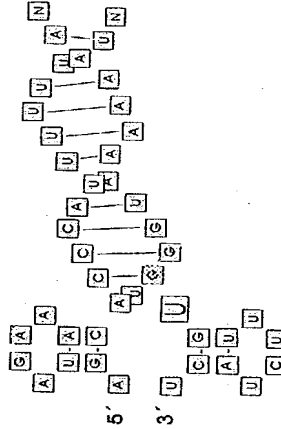
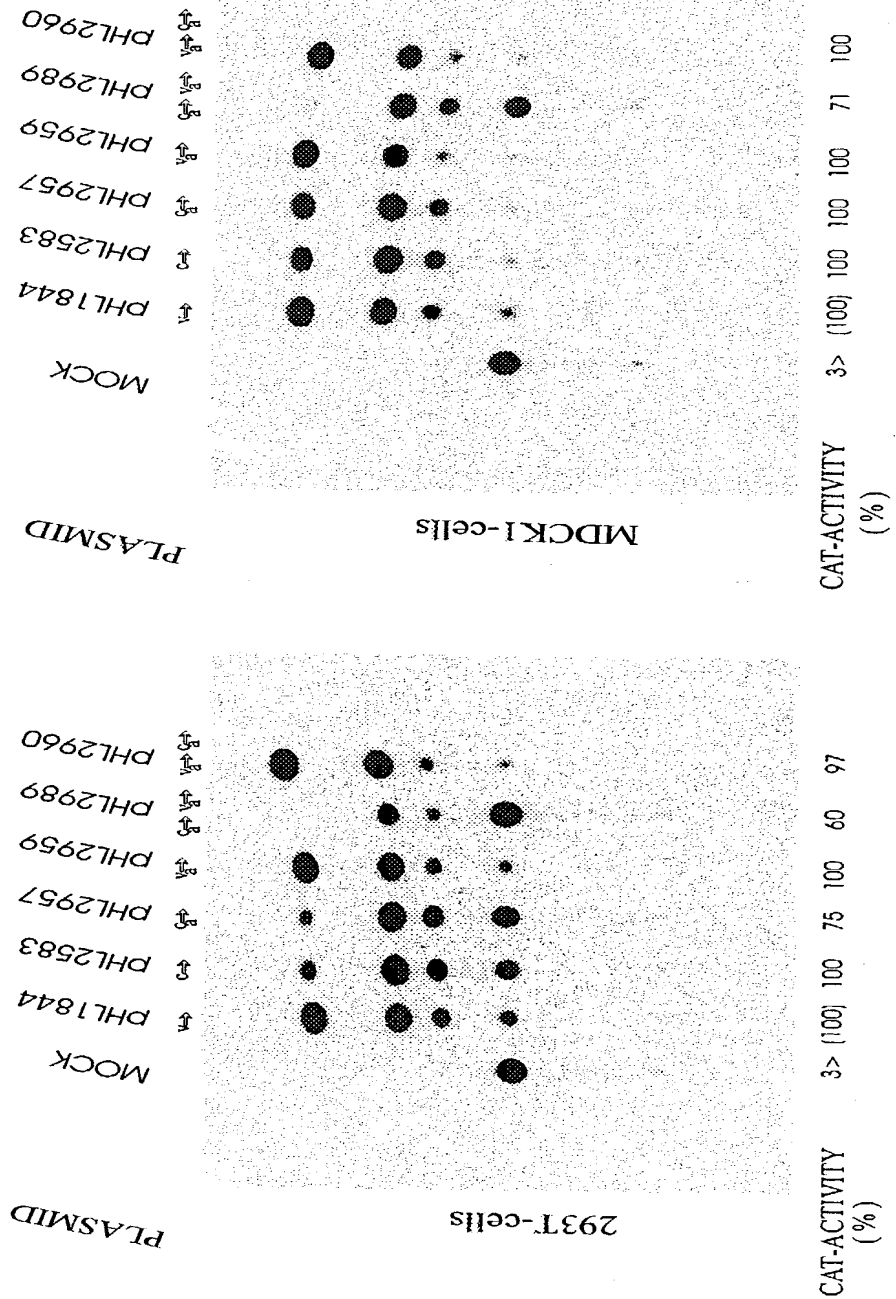


Fig. 10 (continued)



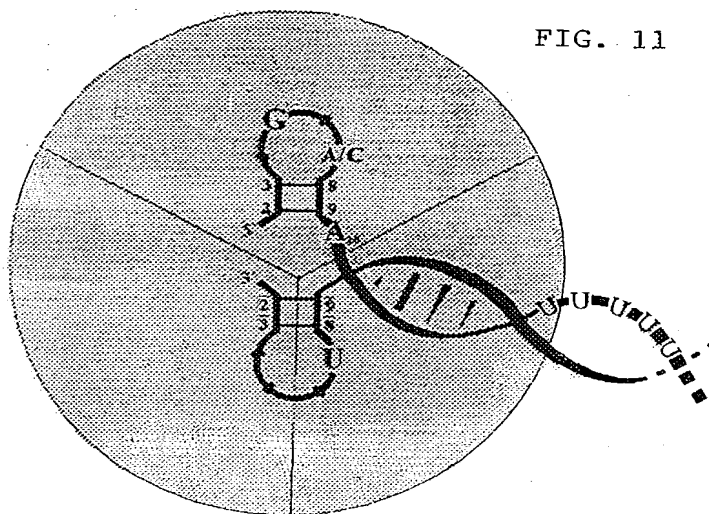
WO 00/53786

12/21

PCT/EP00/01903

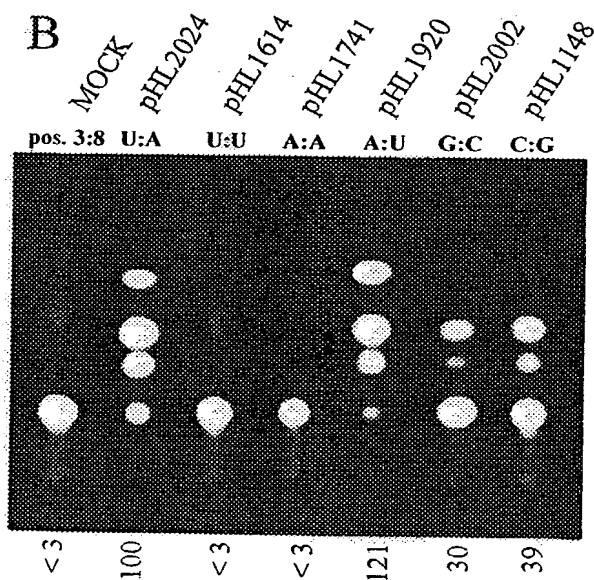
A

FIG. 11



bp-variant position	G - C	A - U	C - G	U - A
2 - 9	pHL2024 100%	pHL1921 41%	pHL2003 < 3%	pHL2004 < 3%
3 - 8	pHL2002 36%	pHL1920 121%	pHL1148 63%	pHL2024 100%
2 - 9	pHL1945 11%	pHL1946 30%	pHL2024 106%	pHL1923 28%
3 - 8	pHL2428 6%	pHL2024 100%	pHL1948 33%	pHL1922 97%

B



C

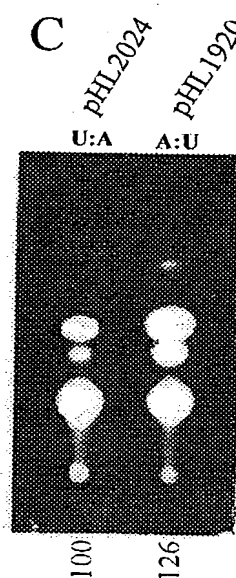
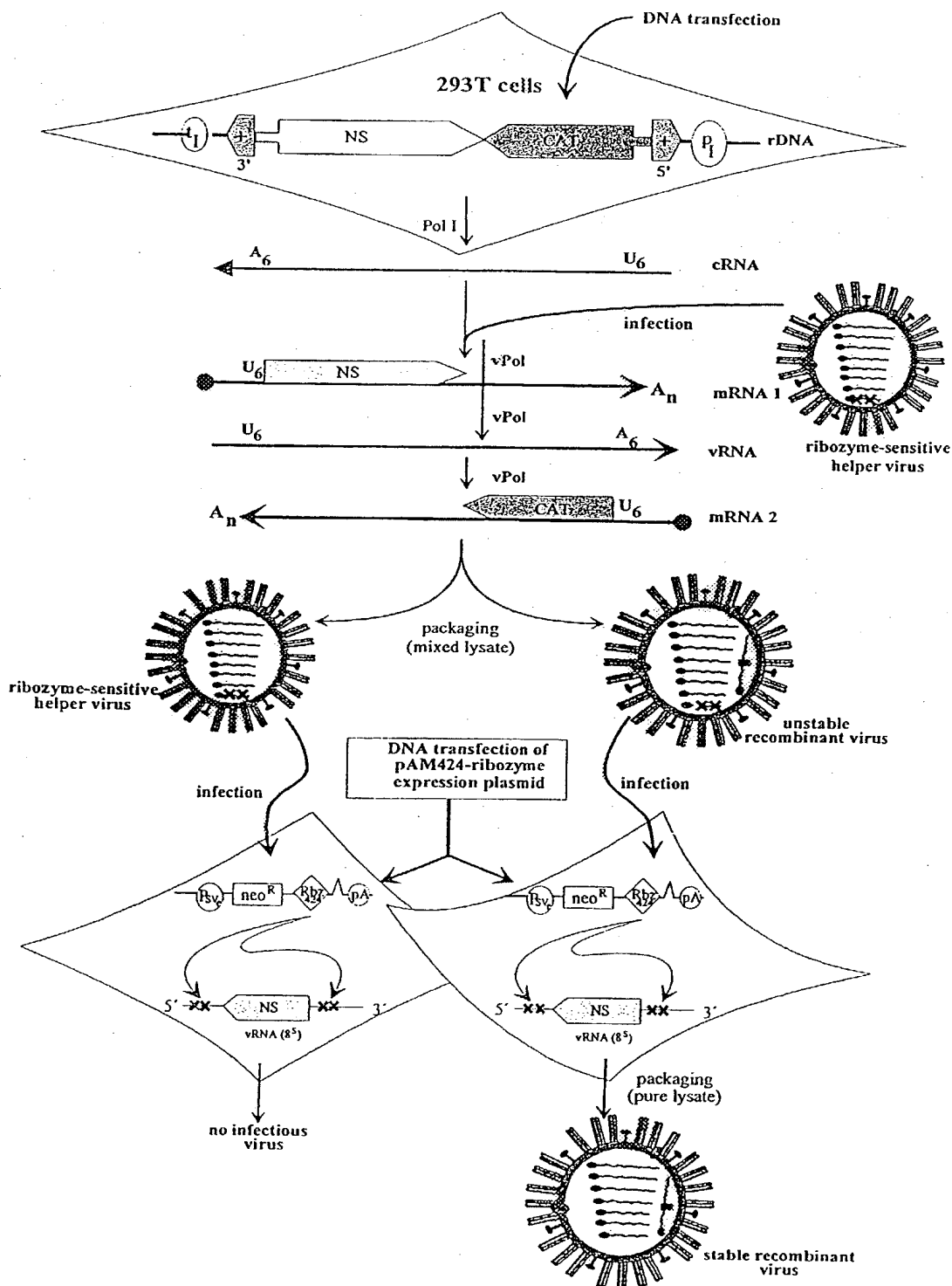


FIG. 12



WO 00/53786

PCT/EP00/01903

14/21

FIG. 13



16/21
FIG. 15

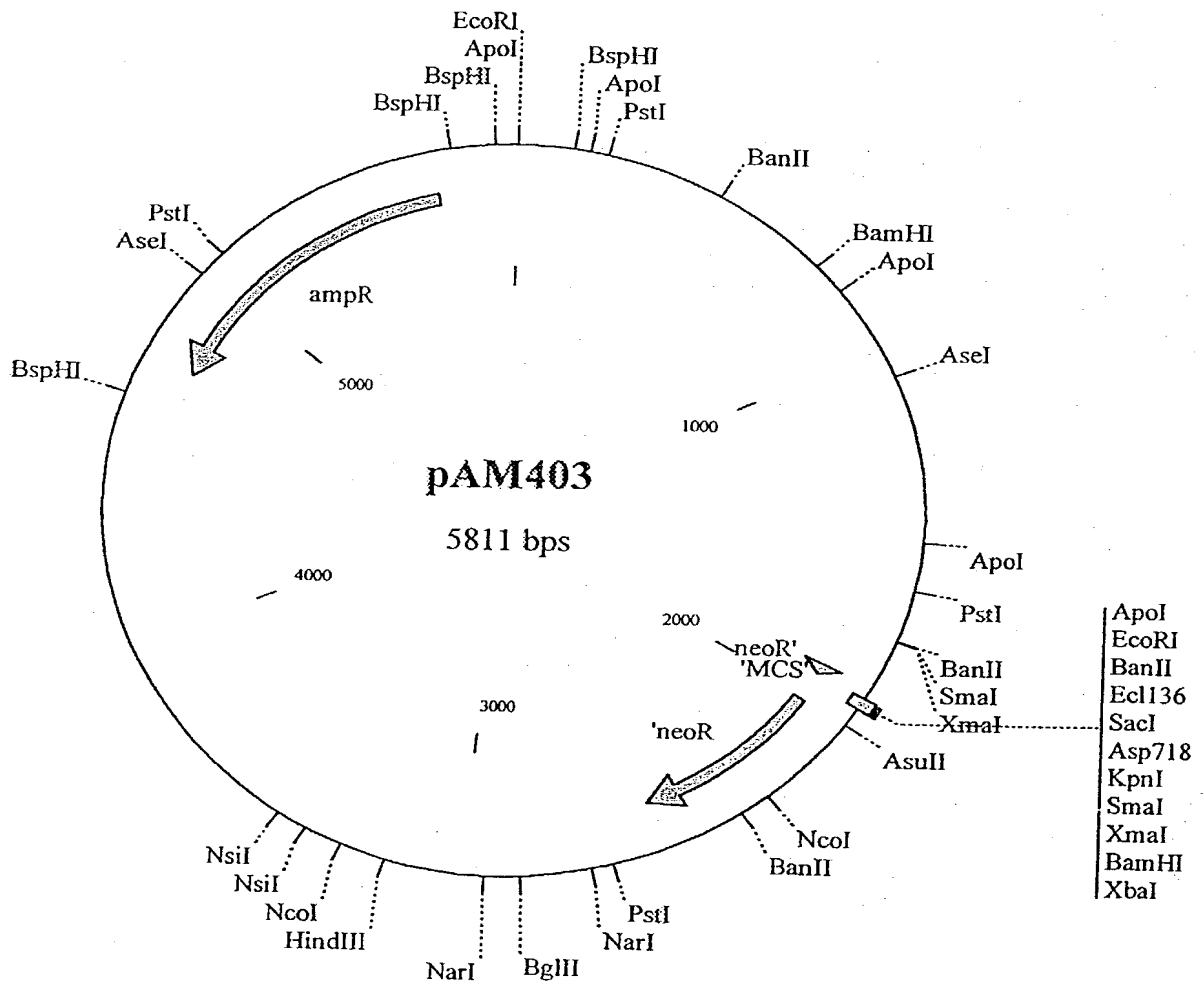


FIG. 16

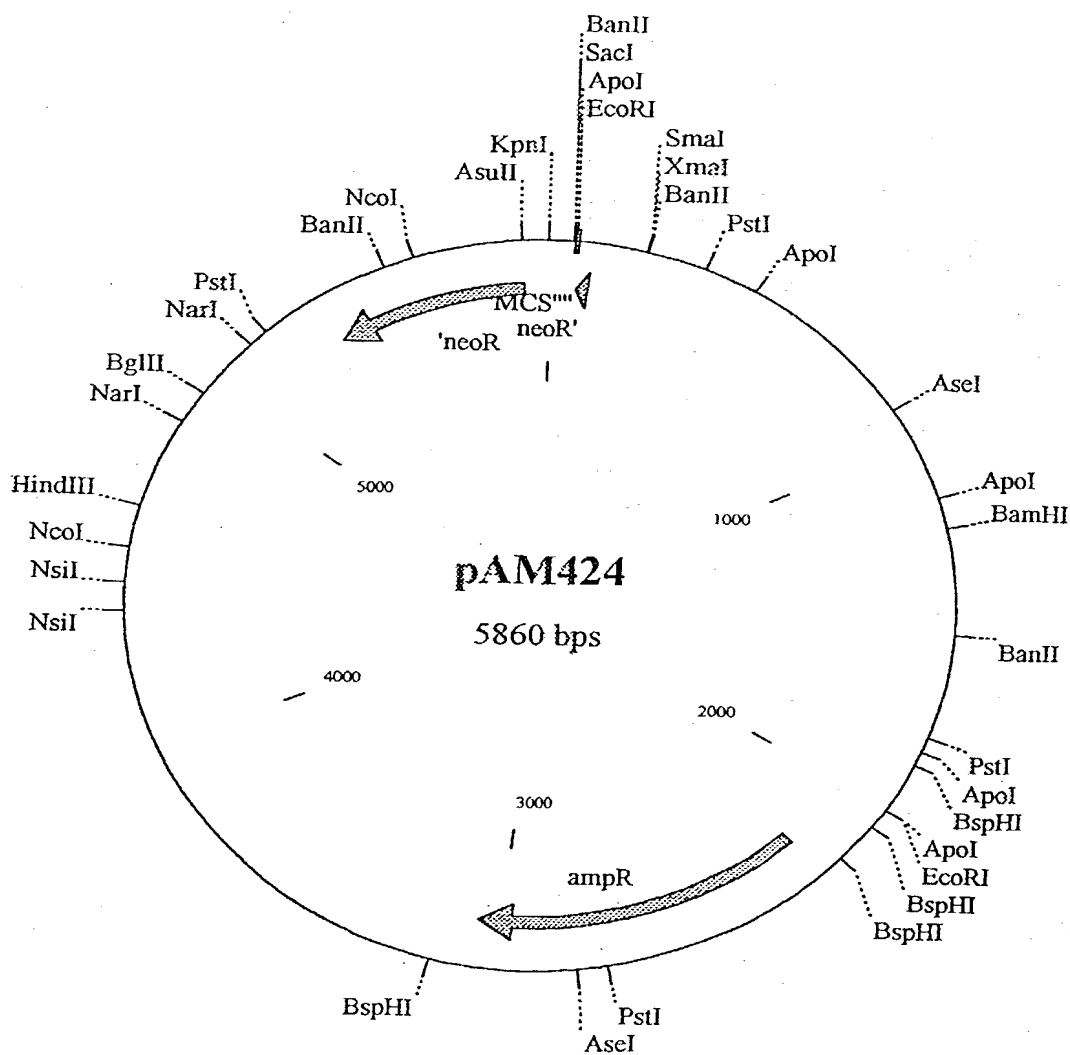


FIG. 17

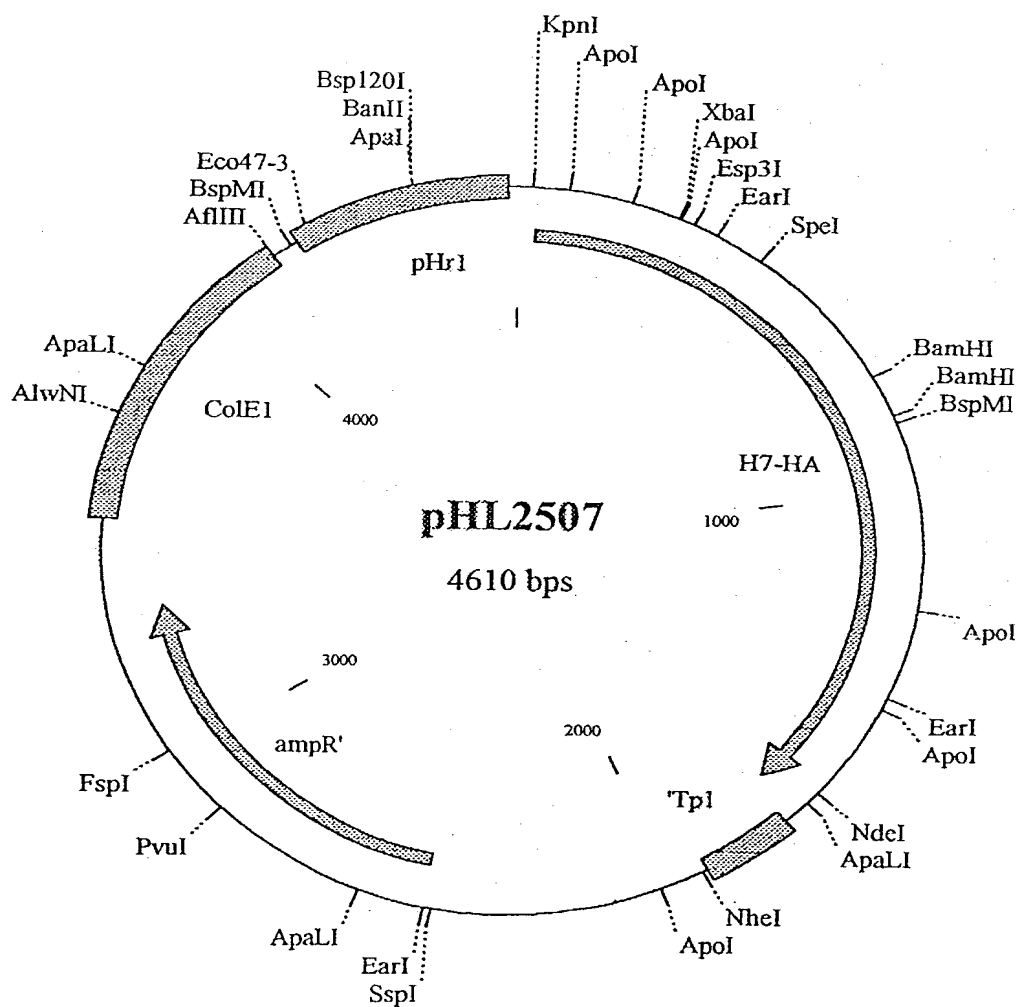
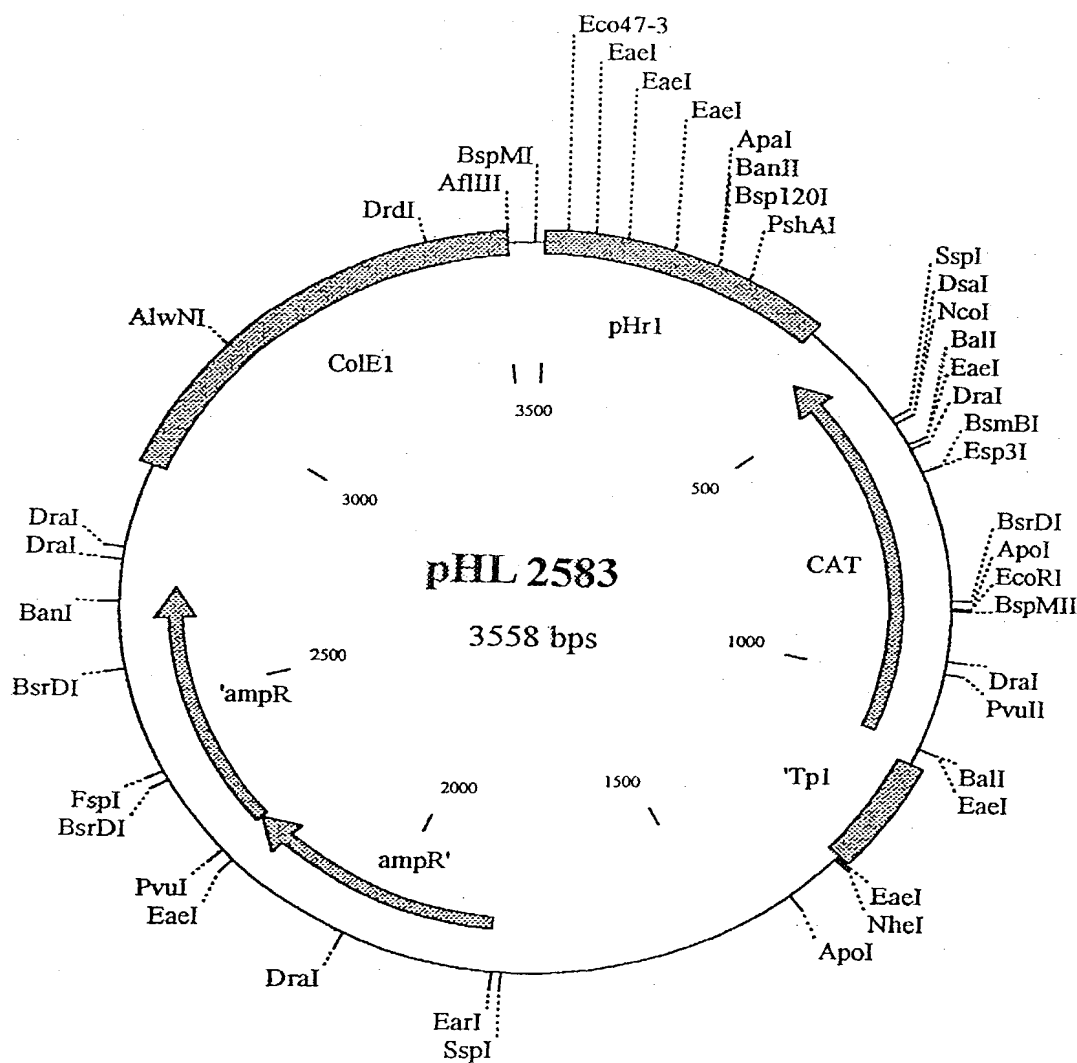


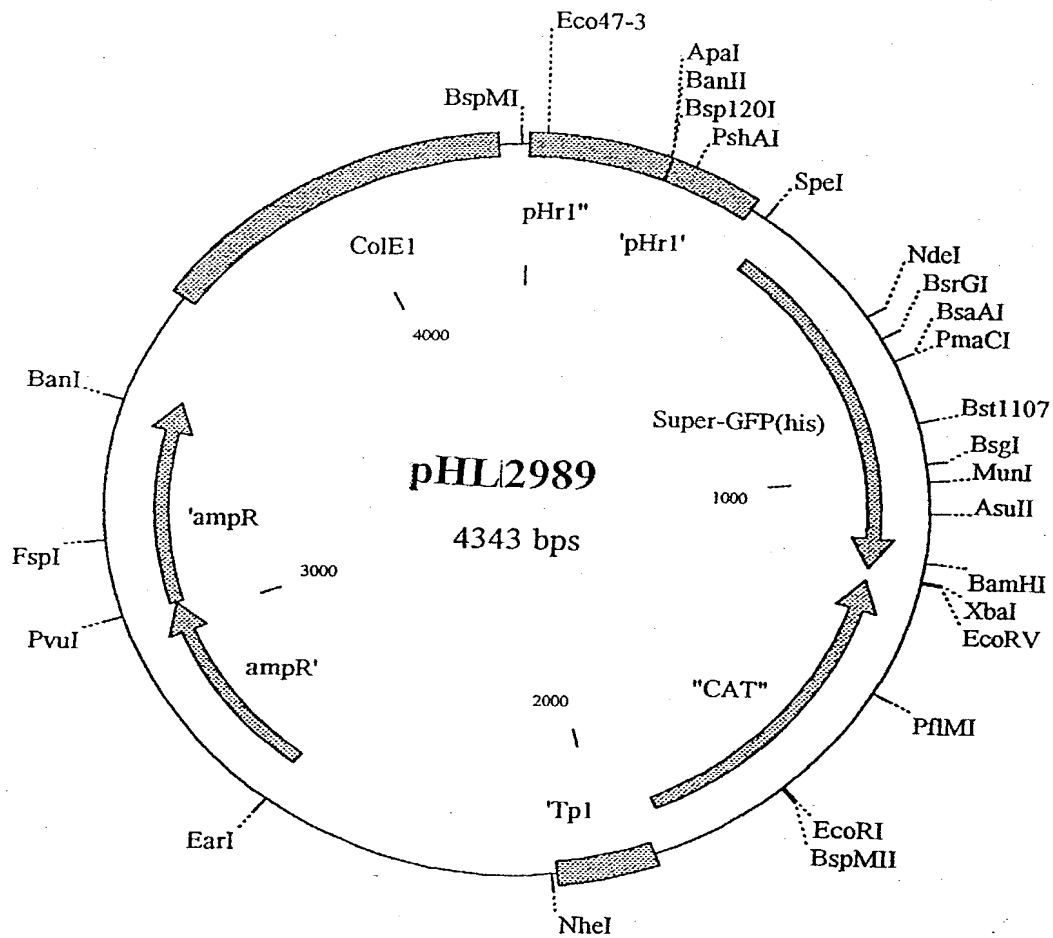
FIG. 18

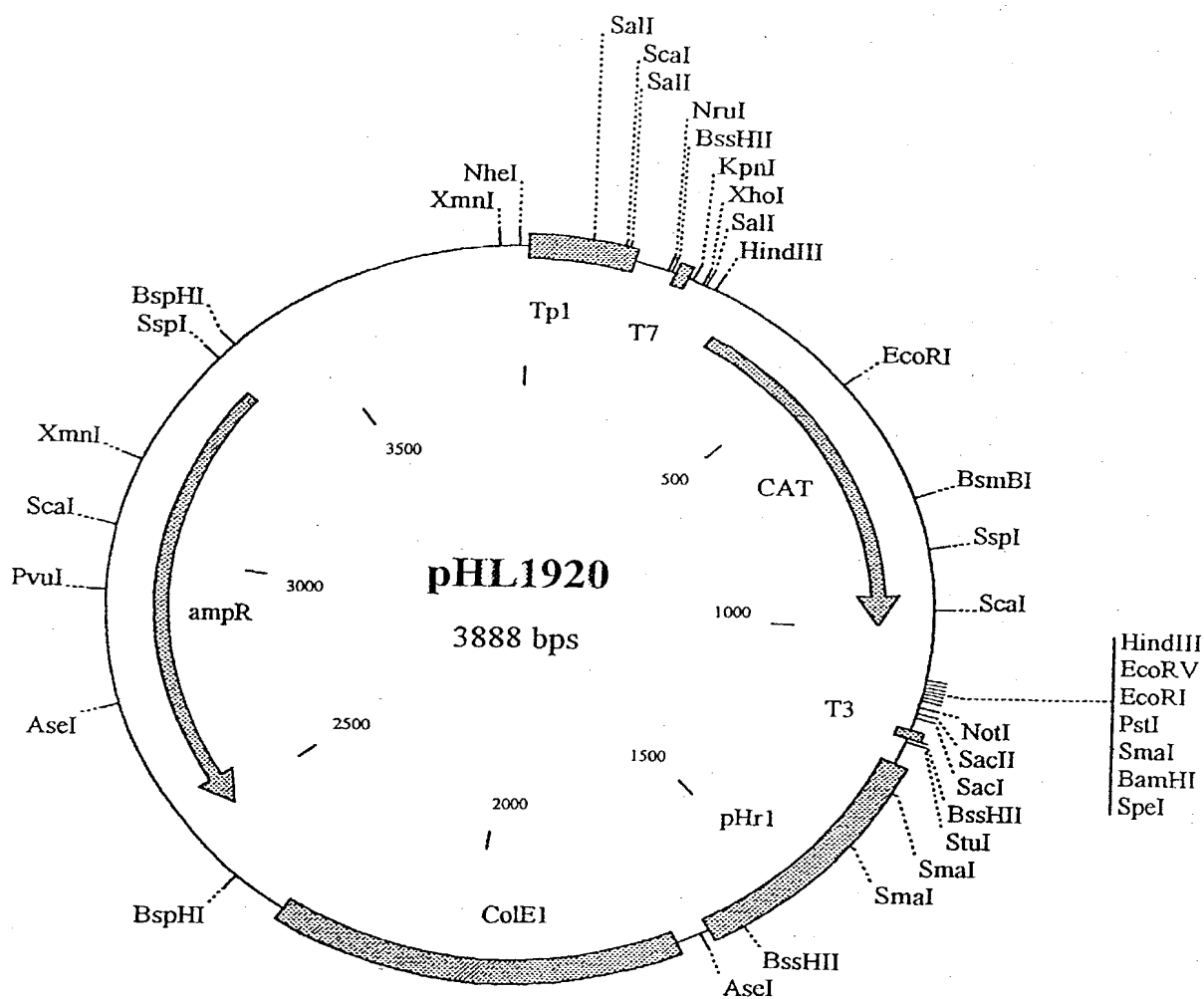


WO 00/53786

20/21
FIG. 19

PCT/EP00/01903





ATTORNEY DOCKET NO.: Kreisler 1098-KGB

COMBINATION DECLARATION & POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name. I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

**STABLE RECOMBINANT INFLUENZA VIRUSES FREE OF
HELPER VIRUSES**

the specification of which was filed on August 31, 2001, as United States Application Serial No. 09/914,658.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations § 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)			<u>Priority Claimed</u>
<u>99104519.6</u>	<u>Europe</u>	<u>6-3-99</u>	<u>x</u> yes <u> </u> no
(Number)	(Country)	(Day/Month/Yr. Filed)	
<u> </u>	<u> </u>	<u> </u>	<u> </u> yes <u> </u> no
(Number)	(Country)	(Day/Month/Yr. Filed)	

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

<u> </u>	<u> </u>	<u> </u>
(Application Serial No.)	(Filing Date)	(Status)
		(patented, pending, abandoned)
<u> </u>	<u> </u>	<u> </u>
(Application Serial No.)	(Filing Date)	(Status)
		(patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punished by fine or imprisonment, or both, under Section

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

Kurt G. Briscoe, Reg. No. 33,141; William C. Gerstenzang, Reg. No. 27,552; Lorimer P. Brooks, Reg. No. 15,155; Bruce Londa, Reg. No. 33,531; Victoria M. Malia, Reg. No. 39,359; and Stephen G. Ryan, Reg. No. 39,015, all of 220 East 42nd Street, 30th Floor, New York, New York 10017; and William R. Robinson, Reg. No. 27,224; Davy E. Zoneraich, Reg. No. 37,267; Mark A. Montana, Reg. No. 44,948 and Robert A. Hyde, Reg. No. 46,354, of 721 Route 202-206, Bridgewater, New Jersey, 08807, my attorneys with full power of substitution and revocation.

SEND CORRESPONDENCE TO:
NORRIS, McLAUGHLIN & MARCUS, P.A.
220 EAST 42nd STREET - 30th FLOOR
NEW YORK, NEW YORK 10017

DIRECT TELEPHONE CALLS TO:
KURT G. BRISCOE
(212) 808-0700

1-00 FULL NAME OF SOLE OR FIRST INVENTOR: Gerd HOBOM
 INVENTOR'S SIGNATURE: [Signature] DATE: 11-09-2001
 RESIDENCE: Arndstrasse 14, D-35392 Giessen, Germany DEX CITIZENSHIP: Germany
 POST OFFICE ADDRESS: Same

2-00 FULL NAME OF SECOND INVENTOR: Ramon FLICK
 INVENTOR'S SIGNATURE: [Signature] DATE: 20.11.01
 RESIDENCE: Lilla Hallsattravägen 28, S-137 93 Västerhaninge, Sweden SE CITIZENSHIP: Germany
 POST OFFICE ADDRESS: Same

3-00 FULL NAME OF THIRD INVENTOR: Anette MENKE
 INVENTOR'S SIGNATURE: [Signature] DATE: 11-09-2001
 RESIDENCE: Ockershäuser Allee 7, D-35037 Marburg, Germany DEX CITIZENSHIP: Germany
 POST OFFICE ADDRESS: Same

4-00 FULL NAME OF FOURTH INVENTOR: Maysa AZZEH
 INVENTOR'S SIGNATURE: [Signature] DATE: 12.11.01
 RESIDENCE: Altenfeldsweg 32, D-35394 Giessen, Germany DEX CITIZENSHIP: Jordan
 POST OFFICE ADDRESS: Same

RECEIVED BY: [Signature] DATE: 11-09-2001
 RECEIVED BY: [Signature] DATE: 11-09-2001
 RECEIVED BY: [Signature] DATE: 11-09-2001

RECEIVED BY: [Signature] DATE: 11-09-2001
 RECEIVED BY: [Signature] DATE: 11-09-2001
 RECEIVED BY: [Signature] DATE: 11-09-2001

099146588 02 Per/09

CRF Errors Corrected by the STIC Systems Branch

Serial Number: 09/914,658B

CRF Processing Date: 1/22/2003
 Edited by: h
 Verified by: h (STIC staff)

ENTERED

- ☐ Changed a file from non-ASCII to ASCII
- ☐ Changed the margins in cases where the sequence text was "wrapped" down to the next line.
- ☐ Edited a format error in the Current Application Data section, specifically: _____
- ☐ Edited the Current Application Data section with the actual current number. The number inputted by the applicant was ☐ the prior application data; or ☐ other _____
- ☐ Added the mandatory heading and subheadings for "Current Application Data".
- ☐ Edited the "Number of Sequences" field. The applicant spelled out a number instead of using an integer.
- ☐ Changed the spelling of a mandatory field (the headings or subheadings), specifically: _____
- ☐ Corrected the SEQ ID NO when obviously incorrect. The sequence numbers that were edited were: _____
- ☐ Inserted or corrected a nucleic number at the end of a nucleic line. SEQ ID NO's edited: _____
- ☐ Corrected subheading placement. All responses must be on the same line as each subheading. If the applicant placed a response below the subheading, this was moved to its appropriate place.
- ☐ Inserted colons after headings/subheadings. Headings edited included: _____
- ☐ Deleted extra, invalid, headings used by an applicant, specifically: _____
- ☐ Deleted: ☐ non-ASCII "garbage" at the beginning/end of files; ☐ secretary initials/filename at end of file; ☐ page numbers throughout text; ☐ other invalid text, such as _____
- ☐ Inserted mandatory headings, specifically: _____
- ☐ Corrected an obvious error in the response, specifically: _____
- ☐ Edited identifiers where upper case is used but lower case is required, or vice versa.
- ☐ Corrected an error in the Number of Sequences field, specifically: _____
- ☐ A "Hard Page Break" code was inserted by the applicant. All occurrences had to be deleted.
- ☐ Deleted **ending** stop codon in amino acid sequences and adjusted the "(A)Length:" field accordingly (error due to a PatentIn bug). Sequences corrected: _____
- ☒ Other: corrected <1807> and <1517> numerical identifiers

*Examiner: The above corrections must be communicated to the applicant in the first Office Action. DO NOT send a copy of this form. 3/1/95